# THE ENZYMATIC HYDROLYSIS OF PYRIDOXINE-5'-BETA-D-GLUCOSIDE IN THE MAMMALIAN SMALL INTESTINE

By

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I would like to dedicate this work to the memory of my grandmother, Marjorie J. Mackey, who contributed significant financial support and unending encouragement throughout my academic career. Her devotion to the educational process and enthusiasm for learning (especially spelling) was an inspiration. I can only hope to pass on as much knowledge to others as she did in and out of the classroom for over 25 years.

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# THE ENZYMATIC HYDROLYSIS OF PYRIDOXINE-5'-BETA-D-GLUCOSIDE IN THE MAMMALIAN SMALL INTESTINE

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An important source of dietary vitamin B-6 is provided by pyridoxine-5'-β-D-glucoside (PNG), a form of vitamin B-6 found in foods of plant origin. While this glycosylated form of vitamin B-6 contributes a significant portion (15%) of daily vitamin B-6 intake, PNG is not completely bioavailable. An obligatory step in the metabolic utilization of PNG is its intestinal hydrolysis by cytosolic PNG hydrolase (PNGH) and lactase-phlorizin hydrolase (LPH). This research was focused on the hydrolytic process of PNG in the mammalian small intestine. Using a rat model, the effect of vitamin B-6 status on PNG hydrolytic activity was examined by feeding either AIN-76A or AIN-93G diets with graded concentrations pyridoxine (PN). Vitamin B-6 deficiency did not increase PNG hydrolysis by PNGH or LPH. Rat growth and PNG hydrolysis were greater in rats fed AIN-93G than rats fed AIN-76A, regardless of dietary PN concentration. LPH catalyzed 50-60% of the total PNG hydrolysis occurring in the small intestine, while PNGH catalyzes 10%. Purification of LPH from rat small intestine confirmed that it was the brush border enzyme that catalyzed the hydrolysis of PNG.

LPH exhibited a $K_m$ of 1.0 mmol/L toward PNG and a Km of 16 mmol/L toward lactose. Lactose was a competitive inhibitor of PNG hydrolysis as both substrates were hydrolyzed at the same active site. In addition to the novel PNG hydrolytic function of LPH, we observed rat LPH to exhibit transferase activity in vitro. Formation of a PN-disaccharide was detected in reaction mixtures containing LPH, PNG, and lactose. Caco-2 cells were also used to examine PNG hydrolysis, absorption, and metabolism. Concentrations of PNG that were $\leq$ 50 μmol/L were absorbed by a saturable mechanism, potentially through a carrier-mediated process. At concentrations >50 μmol/L, PNG was absorbed by passive diffusion. Intracellular concentrations of PNG significantly increased as PNG increased in the media. Increasing concentrations of lactose in the media did not significantly change the intracellular concentrations of PNG; however, intracellular concentration of pyridoxal (PL) significantly decreased. Collectively, these results indicate that 1) vitamin B-6 deficiency does not consistently increase intestinal PNG hydrolytic activity, 2) PNG is hydrolyzed in the small intestine by PNGH and LPH, with most of the hydrolysis catalyzed by LPH, and 3) food selection, i.e., dairy products eaten with fruits and vegetables and individuals with lactose intolerance or lactase insufficiency may reduce PNG bioavailability.

# CHAPTER 1
# INTRODUCTION

Twenty-five years ago, a glycosylated form of vitamin B-6 isolated from rice bran was identified as 5'-O-(β-D-glucopyranosyl)-pyridoxine, or pyridoxine-5'-β-D-glucoside (1). Pyridoxine-5'-β-D-glucoside (PNG) is an abundant source of vitamin B-6 in plant foods and nearly absent in animal-derived foods. PNG contributes a significant proportion of our daily intake of vitamin B-6; however, its nutritional value is limited by its intestinal hydrolysis. For the complete metabolic utilization of PNG, the glycosidic bond needs to be hydrolyzed to release free pyridoxine, which is one form of vitamin B-6 that can be converted to the coenzymatic forms of pyridoxal-5'-phosphate (PLP) and pyridoxamine-5'-phosphate (PMP). PLP and PMP are essential to the normal function of over 100 enzymes that participate in metabolic pathways of nutrients and other biological molecules.

Since the identification of the vitamer, PNG-related research has focused on the nutritional properties of PNG including its bioavailability and metabolism as well as analytical techniques used to measure its concentration in food and other biological materials. Critical to the nutritional significance of PNG is the accurate determination of its concentration in the food supply. Improvements in the analyses of glycosylated and non-glycosylated forms of vitamin B-6 have led to better measurements of PNG concentration in food (2-4). Previous research done by this laboratory has significantly advanced the understanding of PNG bioavailability. *In vivo* studies of PNG bioavailability in rats (5-8), mice, hamsters, guinea pigs (9) and humans (10,11) have shown that the metabolic utilization of PNG is not equivalent to that of PN and that there are species differences in the efficiency of metabolic utilization. The metabolic utilization of PNG is largely determined by its partial hydrolysis and not by the extent of absorption in the small intestine. PNG is hydrolyzed by intestinal β–glucosidases that were reported to exhibit greater activity with decreasing concentrations of dietary pyridoxine (PN) (12,13). The purification and initial characterization of the cytosolic β–glucosidases, broad specificity β–glucosidase and pyridoxine-5'-β–D-glucoside hydrolase (PNG hydrolase), from pig intestine provided *in vitro* evidence of specific intestinal catalysis of PNG hydrolysis by the novel PNG hydrolase, and not BSβG (14).

These investigations served as a foundation for further examination of intestinal PNG hydrolysis and its implications on PNG bioavailability. This laboratory has since conducted investigations that have examined the absorption and hydrolysis of PNG, including the subcellular localization of PNG hydrolytic activities, the effect of dietary PN and other dietary components on intestinal PNG hydrolysis, and the hydrolysis of PNG by the brush border membrane enzyme lactase-phlorizin hydrolase (LPH). The research described in the subsequent chapters has broadened our perspective on the absorption and metabolic processing of PNG in the mammalian small intestine.

## Review of the Literature

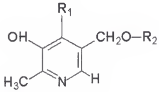
### History

Vitamin B-6 is an essential nutrient to mammalian species as it functions as a coenzyme in several metabolic pathways. The properties of vitamin B-6 were initially established when the vitamin was described as a factor that alleviated and prevented a deficiency in rats known as acrodynia (15). Acrodynia, a B-vitamin deficiency, is a condition diagnosed in rats with symptoms of dermatological lesions and edematous paws and ears (16). Vitamin B-6 was identified from rice bran by Gyorgy in 1935 and further isolated and crystallized three years later by five independent laboratories (17).

## Chemistry

Vitamin B-6 is a term used to describe a family of related compounds having pyridoxine-like activity with the core structure of 2-methyl, 3-hydroxy, 5-hydroxymethyl pyridine. Naturally occurring forms of vitamin B-6 differ in the substituent present on carbon 4 of the pyridine ring (Figure 1-1), which yields the three primary vitamers: pyridoxine (PN), an alcohol; pyridoxal (PL), an aldehyde; and pyridoxamine (PM), an amine. Phosphorylation at the 5'-hydroxymethyl of the pyridine ring forms the respective phosphoric esters: PNP, PLP, and PMP. Pyridoxal-5'-phosphate (PLP) and pyridoxamine-5'-phosphate (PMP) are the predominant forms of the coenzyme found in animal tissues. Vitamin B-6 that is catabolized is converted into 4-pyridoxic acid (4-PA) and excreted in the urine.

Vitamin B-6 also exists as a glycosylated molecule that is synthesized by plants. Naturally occurring forms of glycosylated vitamin B-6 consist of a pyridoxine molecule with one or more monosaccharide or other complex oligosaccharide units attached at the 4' or 5' position via β-glycosidic linkage. The most abundant glycosylated form of dietary vitamin B-6 is pyridoxine-5'-β-D-glucoside, which is comprised of a pyridoxine molecule with one glucosyl group β-linked to the 5' position of pyridoxine.



| $R_1$ | $R_2$ |
|---|---|
| PN= $CH_2OH$ | PN, PL, PM= H |
| PL= CHO | PNP= $PO_3^-$ |
| PM= $CH_2NH_2$ | PLP= $PO_3^-$ |
|  | PMP= $PO_3^-$ |

Figure 1-1. Chemical structures of vitamin B-6.

Vitamin B-6 is soluble in water and stable under acidic pH; however, in alkaline solution, vitamin B-6 is susceptible to degradation by photo-oxidation and heat (18). Both PL and PLP will react with free amino groups, such as α- or ε-amino groups found on amino acids, to form a Schiff base. PLP is well suited as a coenzyme for this reaction since the pyridine ring provides an electron sink for the free electron pair left on the $\alpha$-carbon after the bond between a proton or carboxyl group to the $\alpha$-carbon is broken. The negative charge is stabilized by the PLP ring. This carbonyl-amine reaction is the mechanism by which PLP-dependent enzymes function.

## Functions of Vitamin B-6

Vitamin B-6, most often as PLP, is involved in a host of metabolic reactions including the metabolism of amino acids, lipids, and one carbon units, and the pathways of gluconeogenesis and heme biosynthesis.

Vitamin B-6 is widely recognized for its role in amino acid metabolism. PLP is a coenzyme for aminotransferases, decarboxylases, racemases, and dehydratases. The aminotransferases use PLP in the interconversion of amino acids and their respective $\alpha$-keto acids by forming a stable Schiff base intermediate where PLP becomes an electron sink for negatively charged catalytic intermediates.

The role of vitamin B-6 in lipid metabolism is not clearly defined. Compositional changes in phospholipid were reported to occur in response to changes in dietary concentrations of vitamin B-6 in rats (19,20). More recently, Tsuge and colleagues (21) reported that Wistar rats fed a diet deficient in vitamin B-6 had a 64% reduction in $\Delta6$-desaturase and 80% reduction in acyl CoA oxidase activity compared to the control group. The decreased activity caused an alteration in polyunsaturated fatty acid metabolism, most notably with a reduction in docosahexaenoic acid (DHA). These effects are likely to due to an impaired methylation cycle via S-adenosylmethionine, which decreases phosphatidylcholine and carnitine synthesis.

As reviewed by Selhub (22), vitamin B-6 plays a large role in one-carbon metabolism. PLP serves as a coenzyme for serine hydroxymethyltransferase (SHMT), which catalyzes the interconversion of glycine and serine as well as the formation of methylene tetrahydrofolate from tetrahydrofolate. A metabolic intermediate in the one carbon cycle is the amino acid homocysteine. Homocysteine concentration is maintained, in part, by PLP-dependent enzymatic pathways. The catabolism of homocysteine by the transsulfuration pathway is driven by two PLP-dependent enzymes. The first is cystathionine β-synthase, the enzyme that catalyzes the condensation of serine with homocysteine to form cystathionine. The second enzyme, γ-cystathionase, hydrolyzes cystathionine to release cysteine and α-ketobutyrate (22).

Gluconeogenesis is the metabolic pathway that provides a source of glucose *de novo* from non-glucose precursors. Certain amino acids serve as non-glucose precursors to help maintain concentrations of glucose. The first step in producing glucose from gluconeogenic amino acids is the transamination of the amino acid to form its respective α-keto acid, which is a PLP-dependent process. Another PLP-dependent enzyme involved in carbohydrate metabolism is glycogen phosphorylase. Glycogen phosphorylase catalyzes the cleavage of glycogen to sequentially release glucose-1-phosphate residues that may be converted to the glycolytic intermediate, glucose-6-phosphate. PLP associated with glycogen phosphorylase accounts for a large pool of whole body vitamin B-6. It is estimated that up to 80-90% of body PLP is bound to glycogen phosphorylase (23).

The biosynthesis of heme also requires vitamin B-6 as a coenzyme. Delta-aminolevulinate synthase, a PLP-dependent enzyme, catalyzes condensation of succinyl CoA and glycine to form δ-aminolevulinate, which is the precursor for the porphyrin ring used to synthesize hemoglobin. Deficiency in vitamin B-6 would therefore precipitate a microcytic hypochromic anemia in which erythrocytes have a reduced concentration of hemoglobin. The effects from inherited forms of sideroblastic anemia can be caused by deficiencies in δ-aminolevulinate-synthase (ALAS) activity. The anemia that results form reduced ALAS activity is often overcome with pyridoxine supplementation, if the disorder is clinically classified as pyridoxine-responsive. Mutations in the ALAS2 gene might alter the PLP binding sites on the enzyme (24,25).

There is evidence that vitamin B-6 also functions on a molecular level as a modulator of gene expression. Evidence of transcriptional regulation was provided by Oka and coworkers in rats with vitamin B-6 deficiency (26). Vitamin B-6 deficient rats had a greater abundance of hepatic total poly (A)+ RNA than vitamin B-6 adequate rats; however, the increase was nonspecific and control β-actin levels, which would expectedly remain constant, were similarly increased. Although mRNA levels were not well standardized, Oka and coworkers subsequently reported 5- and 7-fold increases in hepatic glycogen phosphorylase mRNA (27) and albumin mRNA (28), respectively, in vitamin B-6 deficient rats compared to control rats. Sato et al. (29) similarly reported that vitamin B-6 deficiency in rats increased the rate of cystathionase synthesis, which was explained by a several-fold increase in cystathionase mRNA. However, vitamin B-6 deficient rats had essentially the same amount of immunoreactive cystathionase protein as control rats. The authors concluded that vitamin B-6 deficiency upregulated cystathionase synthesis, but also decreased the rate of degradation by lysosomal enzymes.

### Vitamin B-6 Requirements

The Food and Nutrition Board recently revised the requirements for B vitamins, including vitamin B-6. An Estimated Average Requirement (EAR) of 1.1 mg/d and a RDA of 1.3 mg/d for men and women (19-50 y) was established after thorough review of the available literature. These values have been reduced from previous recommendations set in 1989 (Food and Nutrition Board 1989). To determine the requirement, plasma PLP concentration (<20 nmol/L) was used as the major vitamin B-6 status indicator since it best represents tissue stores (Food and Nutrition Board 1998).

As reviewed in the most recent report by the Food and Nutrition Board (1998), vitamin B-6 requirements were typically based on investigations utilizing depletion-repletion protocols with dietary (food) vitamin B-6 in combination with synthetic pyridoxine. Average vitamin B-6 intake as estimated by nationally representative nutrient intake surveys is approximately 1.5 mg/d for women and 2 mg/d for men (Food and Nutrition Board 1998). Although an insufficient dietary intake of vitamin B-6 is not prevalent in the general population, there are significant portions of the population at risk for sub-optimal vitamin B-6 intakes such as pregnant and lactating women (30,31) and the elderly (32).

## Vitamin B-6 in Foods and Supplements

Vitamin B-6 is fairly well distributed throughout the food supply. Foods of animal origin such as meat, fish, eggs, and dairy products are rich in vitamin B-6. Vitamin B-6 exists mostly as PL and PM and their phosphorylated forms (PLP and PMP) in foods of animal origin. Additionally, fortified breakfast cereals and vitamin supplements provide a significant amount of vitamin B-6, as PN, to the typical American diet. Many vegetables and whole grain cereal products are good sources of the vitamin as well. The predominant forms found in foods of plant origin are usually glycosylated. Plant tissues may contain up to 75% of their vitamin B-6 as PNG (2).

Vitamin B-6 loss during cooking and thermal processing is significant; however, it exhibits remarkable stability during storage and handling. The nutritional impact of a reduction in vitamin B-6 content by heating was observed in the 1950s in infants that were formula fed. Coursin reported that infants receiving formula that was thermally processed without subsequent fortification had convulsive seizures along with other neurological abnormalities (33). Neurological based symptoms were alleviated by administration of synthetic pyridoxine to the infants, which prompted the routine fortification of formulas with pyridoxine. The incident led to a number of investigations that determined how the loss of vitamin B-6 by heating occurred. As reviewed by

Gregory (17), the thermal reaction products found in milk were initially thought to reduce the bioavailability of the naturally occurring B-6 vitamers; however, re-evaluation of the evidence found that the thermal processing of the formula destroyed the native vitamin B-6.

Similar to infant formulas, dietary supplements contain pyridoxine hydrochloride as the added form of vitamin B-6. Pyridoxine exhibits the greatest stability among the various B-6 vitamers as it is relatively unreactive with other compounds and is inexpensive to synthesize.

### Pyridoxine Glucosides

Pyridoxine glucosides are just one example of the many vitamin glucosides that occur in nature. Other nutritionally significant glycosylated vitamins, such as vitamin D, niacin, pantothenic acid, and riboflavin have been identified (34).

Glycosylated forms of vitamin B-6 are abundant in foods of plant origin, yet absent in foods of animal origin. The biological significance of the glycosylation of vitamin B-6 in plants is not completely understood, but it is presumed to be a storage form of the vitamin (34). As originally identified in the bran of rice as 5'-O-(β-D-glucopyranosyl) pyridoxine (1) (Figure 1-2), glycosylated vitamin B-6 accounts for a mean 15% of total vitamin B-6 intake in a typical mixed diet (10,30). This percentage could vary depending on food selection and energy intake. Other glucosides of pyridoxine have since been identified. Tadera and colleagues isolated the 4' and 5' isomers of pyridoxine-β-D-glucoside, pyridoxine-5'-β-D-cellobioside, and other conjugated oligosaccharides (35), as well as pyridoxine-5'-β-D-glucoside esterified with malonic or 3-hydroxy-3-methylglutaric acid (36,37), or cellobiosyl-indoleacetate (38). Only beta configurations of pyridoxine glucosides appear to occur naturally in mammalian and plant tissues; however, microbial formation of pyridoxine-α-D-

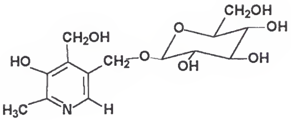


Figure 1-2. Structure of pyridoxine-5'-β-D-glucoside.

glucosides has been reported (39). These α-D-glucoside conjugates are more bioavailable than β-linked PN glucoside, as they are absorbed intact and rapidly taken up and hydrolyzed by the liver (40,41).

Analysis and quantification of pyridoxine glucosides have been reported by a number of investigators. Total glycosylated vitamin B-6 can be determined by the difference in growth responses of yeast before and after treatment with β-glucosidase (2). Alternatively, liquid chromatographic separation methods have been established to measure not only the various forms of vitamin B-6, but also glycosylated variants (4,42,43).

## Bioavailability and Absorption of Vitamin B-6

The bioavailability of vitamin B-6 in humans consuming a mixed diet is approximately 75% (44). As recently reviewed by Gregory (17), vitamin B-6 bioavailability is likely to be reduced by food matrix trapping, nondigestible residue, and only partial utilization of glycosylated forms of vitamin B-6. A glycosylated form of vitamin B-6, specifically pyridoxine-5'-β-D-glucoside, was first thought to be a completely bioavailable form of vitamin B-6 in rats (45). However, later studies found a much lower percentage of PNG (25%) to be effectively absorbed and metabolized in rats (6,8). In everted sacs of rat intestine, PNG was not completely metabolized to the coenzymatic form of PLP (41), indicating that intact PNG was not a metabolically usable form of the vitamin. This is consistent with other investigations examining the bioavailability of PNG in different rodent species (6-9) and humans (10,11). The bioavailability of PNG is much greater in humans (50-60%) than in rats (25-30%), which is thought to be determined by the extent of enzymatic hydrolysis in the small intestine. While PNG can be hydrolyzed in the enterocyte, it also appears in the urine unchanged (absorbed as the intact glucoside). It was estimated that ~35% of dietary PNG appears in the urine in humans, as determined from diet composite analysis of PNG and urinary excretion of PNG (10).

There is evidence that PNG is not hydrolyzed exclusively in the small intestine. In a comparison of oral [$^2H_5$] PN and intravenous (i.v.) [$^2H_2$] PNG doses, it was reported that the i.v. dose of PNG, which bypassed the intestine, was utilized at 28% relative to the oral PN dose. This observation indicated that some hydrolysis of PNG occurred outside of the intestine (10). In rats, less than 5% of an intraperitoneal (i.p.) dose of [$^3$H]PNG was metabolically utilized relative to an i.p. dose of [$^{14}$C]PN (6). However, both PN and PNG were readily absorbed and 93% of the [$^3$H] isotope was excreted in the urine as PNG and its hydrolytic product, PN. Analysis of liver vitamin B-6 revealed a lower degree of metabolism of PNG compared to PN with no retention of [$^3$H]PNG. These observations indicated that PNG was adequately absorbed in the intestine, but poorly utilized, and rapidly excreted into the urine (6). The bioavailability of PNG in other rodent models was also evaluated to assess species relatedness to humans. In an investigation using [$^3$H]PNG and [$^{14}$C]PN, Banks and Gregory (9) reported a 10-34% bioavailability of PNG in rats, 69% in mice, 70% in hamsters, and 92% in guinea pigs. These results suggested that mice and hamsters might be better suited for bioavailability studies applicable to human nutrition.

Intestinal absorption of vitamin B-6 occurs by a nonsaturable, passive process (18). The majority of dietary vitamin B-6 derived from animal sources is in the forms of PLP and PMP. Investigations of PLP absorption, transport, and metabolism have been mostly conducted in intact rat intestine (46-51). There is general agreement that the 5'-phosphate group of dietary PLP is hydrolyzed by luminal alkaline phosphatases, releasing PL that is readily absorbed. PLP can be absorbed across the enterocyte apical membrane intact, although to a lesser extent than its dephosphorylated form (PL) (50). Once absorbed, vitamin B-6 can be phosphorylated by pyridoxal kinase for the purpose of metabolic trapping. While the intestinal absorption of PLP is described as passive and non-saturable, the metabolic phosphorylation of absorbed vitamin B-6 is known to be saturable (52,53). Similar to PLP, PMP also is first acted on by intestinal alkaline phosphatases to yield PM that is absorbed; however, when PMP is present at saturating concentrations, it too will be absorbed with the 5'-phosphate attached (54). Collectively, data from vitamin B-6 absorption studies indicate that the rates of absorption for B-6 vitamers are PL>PN>PM. At physiological concentrations, phosphorylated forms of vitamin B-6 undergo intestinal hydrolysis by alkaline phosphatase prior to absorption. At concentrations well above a physiological dose, PLP and PMP are directly absorbed, with their 5'-phosphate group, although at a rate several fold lower than their respective dephosphorylated compounds (47,54). To cross the basolateral membrane and enter into portal circulation, B-6 vitamers are in a nonphosphorylated form. The intestine does not appear to provide phosphorylated B-6 vitamers to the rest of the organs; rather the liver is responsible for phosphorylation of vitamin B-6 for the distribution to other organs.

### Transport and Metabolism

Vitamin B-6 that is absorbed and metabolized by the intestine enters the portal circulation, predominantly as PL, and is bound to albumin or erythrocytes (55) and is transported to the liver. Although PL and PLP constitute 75-80% of the circulating forms of vitamin B-6 (18), pyridoxine can also be bound and transported by erythrocytes (56). The primary site of vitamin B-6 metabolism is the liver where PLP is generated for use by extrahepatic organs. Nonphosphorylated forms of vitamin B-6 are phosphorylated in the liver by pyridoxal kinase. Pyridoxal kinase, using ATP-Zn+ as a chelated cosubstrate, catalyzes the phosphorylation of the 5' position of PN, PL, and PM to form PNP, PLP, and PMP, respectively (Figure 1-3) (57,58). In addition to the enzymatic phosphorylation function of the liver, FMN-dependent pyridoxamine (pyridoxine) 5'-phosphate oxidase is an abundant enzyme in the liver that is responsible for the conversion of PNP and PMP to PLP (58). This reaction is critical for the metabolism of dietary PN since most tissues have an insufficient amount of oxidase activity to convert PN to PL (59). This enzyme also is of importance because it is tightly regulated by product inhibition. High concentrations of PLP slow the rate of conversion of PNP to PLP, which prevents an excessive accumulation of intracellular PLP.

Catabolites of vitamin B-6 are generated in the liver. Pyridoxamine (pyridoxine) 5'-phosphate oxidase catalyzes the formation of PLP from PNP and PMP, which, in turn, is dephosphorylated to PL. PL in excess is oxidized to the metabolic excretion product, 4-pyridoxic acid (4-PA) by aldehyde oxidase (FAD) or pyridoxal dehydrogenase (NAD), two enzymes that are present in the liver and kidney.

PLP and PL are the predominant circulating forms of vitamin B-6, comprising approximately 75-80% of the total body B-6 (18). As reviewed by Coburn (23), the turnover of vitamin B-6, as PLP, may be described as a five compartment model consisting of muscle, liver, plasma, erythrocytes, and all other pools, which are grouped together into one additional compartment. Evidence from tracer studies calculated total body concentration of vitamin B-6 to be 15 nmol/g and the total pool size in humans is approximately 1000 μmoles (23). This estimation was further supported by data from muscle biopsies from 12 human subjects (60). As reviewed earlier, one of the functions of vitamin B-6 is to serve as a coenzyme for glycogen phosphorylase. Up to 80% of the vitamin B-6 pool, as measured in rats, is associated with glycogen phosphorylase, which

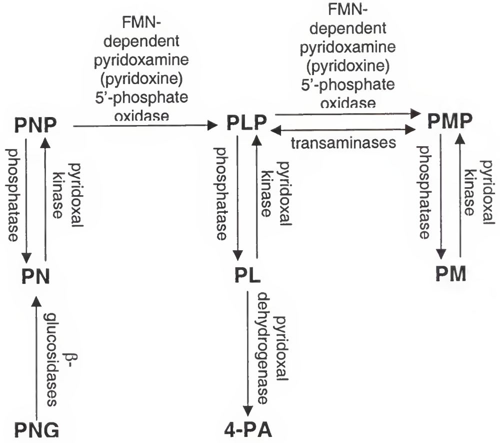


Figure 1-3. Interconversion and metabolism of vitamin B-6. Abbreviations used: PNG (pyridoxine 5'-β-D-glucoside), PN (pyridoxine), PNP (pyridoxine 5'-phosphate), PLP (pyridoxal 5'-phosphate), PL (pyridoxal), 4-PA (4-pyridoxic acid), PMP (pyridoxamine 5'-phosphate), PM (pyridoxamine).

is localized to the muscle (61). The remaining vitamin B-6 is concentrated in the liver, but also distributed to the brain, heart, adrenal glands, kidney, and pancreas (23).

Metabolic utilization of PNG and effects of dietary PNG on the normal metabolism of non-glycosylated B-6 vitamers were examined previously. In rats, a comparison of bioavailability between purified [$^3$H]PNG and alfalfa sprouts that were intrinsically enriched with [$^3$H]PN, forming [$^3$H]PNG, was done in relation to [$^3$H]- and [$^{14}$C]-PN (8). Rats were fed an alginate gel that was extrinsically labeled with [$^{14}$C]PN and purified [$^3$H]PNG or [$^3$H]PN-enriched alfalfa sprouts. This experimental protocol allowed the observation of the metabolic utilization of a purified form of PNG as well as a form that would naturally occur in a plant-derived food in relation to pyridoxine. Rats that were fed the purified PNG received 88% of their vitamin B-6 as PNG. Rats that were fed the alginate gel with the alfalfa sprout homogenate added consumed ~45% of their vitamin B-6 as PNG. Control rats consumed >80% of their vitamin B-6 as PN. Isotopic ratios of [$^3$H] / [$^{14}$C] dose in the liver, carcass and plasma were significantly lower in the rats fed any form of PNG than control rats that were fed PN. Conversely, the relative isotopic ratios in the urine and feces were significantly higher than controls. These data indicated that the absorption and metabolic utilization of PNG was incomplete. Dietary PNG was not retained in the liver as PMP, PLP, or PL. Significantly more PNG was excreted in the urine and feces than PN. There was no [$^3$H]PNG detected in the livers of rats fed the glucoside (8). Further evaluation PNG bioavailability was done in rats either PN or PNG as their sole source of vitamin B-6. Changes in vitamin B-6 status were assessed using erythrocyte aspartate transaminase (EAST) stimulation, plasma PLP concentration, and urinary 4-PA excretion in response to the form of dietary vitamin B-6 (7). Rats consuming their vitamin B-6 as PNG had an elevated stimulation of EAST with PLP, lower plasma PLP concentrations, and a diminished 4-PA excretion compared to rats consuming their dietary vitamin B-6 as PN. This same group of investigators also examined the metabolic utilization of PNG compared to PN by using isotopically labeled [$^3$H]PNG and [$^{14}$C]PN given either as an oral or intraperitoneal dose to vitamin B-6 adequate or deficient rats (6). Rats injected with both [$^3$H]PNG and [$^{14}$C]PN excreted twice the amount of [$^{14}$C]-labeled 4-PA when fed a diet adequate in vitamin B-6 compared to a diet deficient in vitamin B-6. In contrast, rats fed a diet containing no vitamin B-6 excreted the same amount of [$^3$H]-labeled 4-PA as the rats fed the vitamin B-6 adequate diet, with 93% of the label excreted as PN and PNG. The increased urinary clearance of [$^3$H] indicated the reduced utilization of PNG relative to PN and that a deficiency in vitamin B-6 did not improve the metabolic processing of PNG. Additionally, hepatic vitamin B-6 metabolites detected as [$^{3}$H] or [$^{14}$C], were not significantly different between adequate and deficient rats, regardless of route of administration. Although the retention of [$^{3}$H]-labeled B-6 vitamers was significantly less in the liver and carcass as compared to [$^{14}$C]-labeled vitamers, PNG appeared to undergo partial hydrolysis and act as PN in the metabolic interconversion of hepatic vitamin B-6 (6). The results from these studies showed that PNG is not as efficiently metabolized as PN and that vitamin B-6 deficiency reduces vitamin B-6 catabolite clearance, i.e., a reduction in 4-PA excretion.

Gilbert and Gregory (5) investigated the effect of dietary PNG on the metabolic utilization of co-ingested PN in rats using radiolabeled [$^{14}$C]PN and unlabeled PNG. Administration of PNG concurrently with PN antagonized the utilization of PN as observed through the altered distribution of hepatic vitamers and reduced retention of labeled PN. Of particular interest was the direct linear relationship found between the dose of PNG and the appearance of [$^{14}$C]PNP, which was later supported by Nakano and Gregory (13), who also reported that as dietary PNG increases in rats, liver PLP decreases. Since dietary PNG interfered with the normal production of hepatic PLP, Gilbert and Gregory (5) hypothesized that PNG had an inhibitory effect on pyridoxamine (pyridoxine) 5'-phosphate oxidase. However, further investigation of the inhibitory effects of PNG on purified pyridoxamine (pyridoxine) 5'-phosphate oxidase and found no effect on the activity *in vitro* (62). These results were corroborated by Zhang et al. (63). In liver cell homogenates, [$^{3}$H]PN incubated in the presence of PNG at either 0.5 or 5.0 μmol/L did not significantly alter the specific activity of pyridoxal kinase or pyridoxamine (pyridoxine) 5'-phosphate oxidase. The authors concluded that an increased liver PNP was the result of PNG competitively inhibiting the uptake of PN in isolated hepatocytes. The formation of PLP from PNP proceeds through the requisite phosphorylation of PN to PNP, which is then oxidized to PLP. Without an adequate supply of PN to the liver, the amount of PLP formed from PNP is reduced. Zhang and coworkers also calculated the cellular uptake and metabolism of PNG relative to PN, which was 20 and 0.2%, respectively (63).

Effects of dietary PNG on the metabolism of PN also have been investigated in humans. Hansen and coworkers monitored vitamin B-6 status in response to feeding women diets with varying concentrations of PNG (64). Nine healthy women were fed diets that were designated as either high or low in PNG for 18d each in a crossover design. Vitamin B-6 nutritional status was assessed by using plasma and erythrocyte PLP, erythrocyte aspartate and alanine aminotransaminase (EAST and EALT) stimulation, urinary 4-PA and total B-6 fecal excretion as indicators. The diet providing 27% of total vitamin B-6 as PNG (high PNG diet) yielded a 10% lower urinary 4-PA, 12% lower fecal vitamin B-6 excretion, and 18% and 17% lower plasma and erythrocyte PLP, respectively, than the diet providing 9% total vitamin B-6 as PNG (low PNG diet). The EAST and EALT stimulation tests were similar between the two diets. Similarly, Nakano et al. (11) observed a decline in 4-PA excretion in human subjects given increasing doses of [$^2H_2$]PNG. Rats are less susceptible than humans to the antagonistic effects of PNG on PN.

### Cytosolic Pyridoxine-5'-β-D-glucoside Hydrolase (PNG Hydrolase)

Prior to the purification and identification of the cytosolic PNG hydrolase from pig intestinal mucosa, PNG was presumed to be hydrolyzed in the small intestine by another soluble β-glucosidase, broad specificity β-glucosidase (BSβG) (6,8,12,13). To better understand the hydrolysis of PNG by BSβG, McMahon and coworkers purified and partially characterized not only intestinal BSβG, but also a novel cytosolic β-glucosidase, pyridoxine-5'-β-D-glucoside hydrolase from pig jejunum (14). BSβG is a soluble enzyme purified from guinea pig and human liver that has an approximate molecular weight of 50-60 kD (65-67). The cDNA encoding this protein has been cloned and sequenced from the guinea pig liver (68). While BSβG does not hydrolyze PNG (14) or other nutritionally significant oligosaccharides, it displays activity for a wide range of substrates, with particular specificity to aryl aglycones (69,70).

Cytosolic PNG hydrolase is a soluble β-glucosidase with an acidic pH optimum (5.5) and a molecular mass of 130 kD (denatured) and 160 kD (native). In contrast to BSβG, PNG hydrolase does not hydrolyze aryl glucosides but exhibits hydrolytic affinity for PNG, cellobiose, and lactose. PNG hydrolase was characterized to have a $K_m$ of 0.88 ± 0.12 mmol/L and a $V_{max}$ of 13.2 ± 0.8 μmol/h*mg protein for PNG. Inhibition experiments found that PNG hydrolase, like other β-glucosidases, was inhibited by conduritol B epoxide and glucono-δ-lactone. Hydrolytic activities of both PNG hydrolase and BSβG are affected by sulfhydryl reagents, which implicated cysteine residues in the catalysis at the active site.

There is remarkable amino acid homology between short amino acid sequences obtained from cytosolic PNG hydrolase (~95-99%) purified from pig intestine and the brush border enzyme, lactase phlorizin hydrolase (LPH) (Tseung, et al., unpublished) as analyzed by the BLAST alignment procedure (71). All of the short sequences aligned with rat, rabbit, and human LPH. Five of these sequences (total of 51 amino acids) also aligned with regions of the precursor sequence of LPH (aa 20-866) that are not present in the mature LPH enzyme. However, there is a region in the precursor sequence of LPH (aa 458-806) where no aligning sequences from cytosolic PNG hydrolase were found. This might indicate that cytosolic PNG hydrolase is a splice variant derived from the LPH gene. It is also well known that substantial sequence homology exists among β-glucosidases, so LPH and PNG hydrolase might be products of related, but distinct genes.

## Lactase Phlorizin Hydrolase

The digestion of dietary carbohydrates relies on the disaccharidases present in the small intestine. Perhaps the most important carbohydrate-digesting enzyme to the developing mammalian species nourished via a milk-based diet is the intestinal brush border enzyme lactase phlorizin hydrolase (lactase). Lactase phlorizin hydrolase (EC 3.2.1.23, 62, and 108) (LPH) is a membrane bound glycoprotein in the intestine that is primarily responsible for the hydrolysis of dietary lactose into its constituent monosaccharides glucose and galactose. LPH was previously purified and characterized by several laboratories from the small intestine of different species (72). Lactase phlorizin hydrolase is named for its two distinct hydrolytic activities: one for the hydrolysis of lactose and other hydrophilic β-glucosides (lactase), and the other for the hydrolysis of aryl- or alkyl-glucosides, such as β-glucoceramides (phlorizin hydrolase) (73-75). Wacker and colleagues assigned the lactase activity to glutamate$_{1273}$ and the phlorizin hydrolase activity to glutamate$_{1749}$ (75). The LPH gene (LCT) was localized to chromosome 2 and cDNA cloning led to the translation of the LPH sequence to 1927 amino acids (72). The enzyme is synthesized as a single polypeptide glycosylated precursor of 215 kDa (pro-LPH), which undergoes folding to form a homodimer; a step that is critical for membrane targeting and enzymatic activity (76,77). Transport of the homodimer through the Golgi apparatus yields a complex glycosylated form of the protein (230 kDa) (78). Subsequently, the protein undergoes two proteolytic cleavages. The first occurs intracellularly at Arg$_{734}$, which yields a transport-competent form of the enzyme. The second cleavage, occurring after insertion in to the microvillar membrane, is catalyzed by pancreatic trypsin at Arg$_{868}$, which generates the mature form of LPH (130-160 kDa) (79,80). The expression of LPH is greatest in the jejunum. Immunohistochemical and in situ hybridization detection methods in several species revealed that LPH expression increases from crypt to mid-villus, and declines at the villus tip (77,81,82).

The control of LPH expression has been studied extensively in light of the connection with the post-weaning decline in lactase activity that is observed in maturing mammals. Current opinion on the decline of lactase activity focuses on transcriptional control of the LPH protein (77). This is supported by data that positively correlate levels of mRNA and LPH activity in rats (81,83,84), pigs (85), and sheep (86). In spite of the compelling evidence of transcriptional control, there are reports of secondary, or post-translational control of LPH expression. Using [$^{13}$C]- or [$^{3}$H]-leucine and [$^{13}$C]-phenylalanine, Dudley and coworkers have measured the brush border fractional synthesis rate (FSR) of intestinal disaccharidases (87), including isoforms of LPH (88) to assess dietary effects on intracellular processing of LPH (85,89,90). The ratio of the isotopic enrichment of an LPH glycosylated precursor and the enrichment of a mature LPH isoform yields the FSR for LPH (87). Changes in LPH isoform labeling, (FSR), in response to a dietary treatment, e.g. protein deficient diet (85), is indicative of changes in post-translational modification and further, the regulation, of mature LPH expression. This procedure is validated by a strong relationship between the relative abundance of the LPH isoforms and developmental differences in LPH activity (91). Using this methodology, post-translational control of LPH expression was reported to be reduced in protein malnutrition (85,90) and increased by administration of insulin-like growth factor-I (IGF-1) (92,93) and colostrum (89)

A reduced expression and activity of LPH in humans most often occurs during the transition from a predominantly milk-based diet to a diet of solid food. Lactase decline occurs somewhere between the ages of 5-7, but as late as 20 years (94). In humans, the normal physiological decline in LPH activity is termed adult-onset lactase decline and is more commonly known as lactase deficiency or lactose intolerance. It is usually recognized by the variety of abdominal and intestinal related symptoms that are caused by the osmotic effect of undigested lactose present in the lumen. Although nearly all mammals are born with lactase, some infants are born with a rare congenital lactase deficiency.

Initial clinical characterization of intestinal disaccharidases, including β-galactosidases, as well as their deficiencies were reported in a series of papers by Arne Dahlqvist and coworkers (95). Dahlqvist is also credited with the development of a rapid and accurate determination of lactase activity as measured by the release of glucose using glucose oxidase and a colorimetric agent (96). The condition of lactose intolerance can be diagnosed by assessing the extent of lactose digestion. Lactase activity can be directly measured by intestinal mucosa biopsy or by an intestinal perfusion method that pumps lactose into the intestine and measures glucose release by sampling the contents of the lumen (97). Although these methods are considered to be the most accurate, they are used infrequently and are the most invasive. More convenient tests used in the routine diagnosis of lactose intolerance include breath tests, blood sampling, and urine analyses (98). Traditionally, breath hydrogen concentrations are used to detect lactase deficiency. This test measures the release of $H_2$ in the breath after a dose of lactose. Undigested lactose is fermented by colonic microflora, which produces hydrogen gas that is absorbed into portal circulation and later expired in the breath (99). Similarly, breath$^{13}CO_2$ can be measured after ingestion of [$^{13}$C]lactose. Determination of blood glucose or galactose concentrations following a dose of lactose can also be used to diagnose lactose intolerance. More recently, it was suggested that the ratio of [$^{13}$C]/[$^{2}$H]-glucose in blood after ingestion of [$^{13}$C] lactose and [$^{2}$H] glucose was a more accurate way to assess lactose digestion (100). Low concentrations of galactose in the urine following a dose of lactose may also diagnose lactose intolerance.

The prevalence of lactose intolerance differs globally. Worldwide, it is estimated that 75% of the adult population is lactase deficient. In the United States, the lowest prevalence is among whites of Western European descent (15%) and increases to 80% in the African-American and 90% in the Asian-American population. In Asian countries, the percentage of lactose intolerance is at least 50% and varies among ethnicities (101). It is proposed that the persistence of lactase activity, rather than the loss, is abnormal. As reviewed by Vesa, hypotheses to explain lactase persistence were derived from cultural practices and natural genetic selection (98). At the advent of dairy farming, populations that had higher levels of lactase activity would exhibit an evolutionary advantage for survival in times of dietary scarcity. Today, the highest prevalence of lactase persistence is observed in individuals that are descendents from areas with a long history of dairy farming, as in those from Western Europe. Genetic markers of physiological decline in lactase activity were recently identified by examining variants in the lactase (LCT) gene (102). Seven polymorphic microsatellite markers flanking the LCT gene in nine Finnish families (n=196) with adult-onset hypolactasia were analyzed. The individual family members were biochemically verified to be lactose intolerant or tolerant. Two DNA variants that were upstream of LCT were identified: C/T-13910 and G/A-22018 with the C and G alleles associated with lactase deficiency. Individuals that were diagnosed with lactase deficiency (n=59) were all homozygous (CC) with respect to the C/T variant and 6 were heterozygous (GA) and 53 homozygous (GG) with respect to the G/A variant. None of the lactase persistence subjects (n=137) were homozygous with respect to the C or G alleles. Subjects (n=74) were TT with respect to the C/T variant and AA with respect to the G/A variant. The remaining 63 were heterozygous at both positions. The authors speculated that the variant identified in all of the lactose intolerant individuals is the original form of the gene and that it mutated to acquire the ability to hydrolyze lactose. The prevalence of CC genotype among different populations was also examined. White North Americans had the lowest prevalence (7.6%), followed by the French (41%) and black North Americans (79%). These data are not only consistent with current epidemiological estimates of prevalence of lactose intolerance, but also propose another potential method of screening individuals for the diagnosis of lactose intolerance.

In summary, the preceding review of the literature discussed the nutritional significance and bioavailability of PNG as an important form of dietary vitamin B-6. Intestinal β-glucosidases catalyze the intestinal hydrolysis of PNG, which is the limiting factor of PNG bioavailability. The following research sought to examine the bioavailability of PNG in mammals with particular focus on its intestinal hydrolysis.

## Hypotheses

The research described in the subsequent chapters was based on the existing literature and preliminary studies done to describe the hydrolysis and metabolism of PNG. The present studies were done to test the hypotheses:

1) Dietary vitamin B-6 deficiency increases the PNG hydrolytic activity in the mammalian small intestine.
2) The brush border membrane enzyme, lactase phlorizin hydrolase, catalyzes the hydrolysis of pyridoxine-5'-β-D-glucoside

## CHAPTER 2

## HYDROLYTIC ACTIVITY TOWARD PYRIDOXINE-β-D-GLUCOSIDE IN RAT INTESTINAL MUCOSA IS NOT INCREASED BY VITAMIN B-6 DEFICIENCY: EFFECT OF BASAL DIET COMPOSITION AND PYRIDOXINE INTAKE

### Introduction

Pyridoxine-5'-β-D-glucoside (PNG), a glycosylated form of pyridoxine provides a significant dietary source of vitamin B-6. This form of vitamin B-6 was first isolated from rice bran (1) and can be found in foods of plant origin (2,18,42). PNG accounts for 5-75% of the vitamin B-6 present in plant tissues. In a mixed American diet, it is estimated that half of the dietary vitamin B-6 intake is from plant sources (103). Although PNG provides approximately 15% of the total vitamin B-6 in typical diets in the United States (30,64), some patterns of food selection could lead to a much larger proportion of dietary vitamin B-6 as PNG.

The bioavailability of PNG in humans is 50-60% relative to pyridoxine, (10,11) which is greater than that estimated for rats (25-30%) (6-9). The rate-limiting factor in the utilization of PNG as a source of vitamin B-6 is the enzymatic hydrolysis of the β-glucosidic bond, rather than the intestinal absorption of intact PNG or free PN derived from PNG hydrolysis (4,8). The variations in PNG bioavailability within and among species may be explained by differences in intestinal enzymatic activities toward PNG. Cytosolic broad specificity β-glucosidase (BSβG) (66) was initially thought to be responsible for the hydrolysis of dietary PNG (12,13,104). However, purification and kinetic analysis of BSβG from pig intestine revealed that this enzyme does not hydrolyze PNG (14). This observation led to the detection and purification of a novel intestinal cytosolic β-glucosidase, designated pyridoxine-5'-β-D-glucoside hydrolase (PNG hydrolase), that catalyzed the hydrolysis of PNG. Recent data from this laboratory indicate that PNG can be hydrolyzed in both the cytosolic and membrane subcellular fractions of rat small intestinal mucosa. In an investigation of the age-dependent enzymatic hydrolysis of lactose and PNG, Armada et al. (105) measured PNG hydrolysis in the cytosolic and brush border membrane subcellular fractions of intestinal mucosa in nursing, weaned, and adult rats. A concurrent publication by Mackey et al. (106) reported the kinetic analysis of PNG hydrolysis as catalyzed by rat brush border membrane and purified lactase-phlorizin hydrolase (LPH), a β-glucosidase in the intestinal brush border membrane. Although these investigations reported PNG hydrolysis to be catalyzed in both subcellular compartments of the intestinal mucosa, the contribution of these cytosolic and brush border membrane enzymes toward PNG hydrolysis *in vivo* is not fully known.

Previous investigations of the effects of vitamin B-6 nutritional status on cytosolic PNG hydrolase activity in the small intestine of guinea pigs and rats showed that PNG hydrolase activity was inversely related to vitamin B-6 status (12,13). Subsequent studies (Mackey et al., unpublished) examined more closely the effect of vitamin B-6 nutritional status on the activity of cytosolic PNG hydrolase in rat intestine and showed that vitamin B-6 status had little or no reproducible effect on this enzymatic activity. This raised the question of whether differences in the basal diet may have had an effect on the vitamin B-6 status of the rodents and perhaps intestinal β-glucosidase activity. Our preliminary rat studies that did not find an effect of dietary vitamin B-6 on intestinal β-glucosidase activity used AIN-93G purified diet instead of the AIN-76A diet that was used in previous studies (12,13). Although there are several differences between the two diet formulations (107), we hypothesize that the carbohydrate in the AIN-93G, mostly cornstarch, is a more fermentable carbohydrate for the microflora of the cecum and large intestine than sucrose, which is the major carbohydrate component of the AIN-76A formulation. In the presence of fermentable carbohydrate, the gut microflora actively synthesize protein, growth factors, and other nutrients, including vitamin B-6 (108), which may enhance the vitamin B-6 nutritional status of the rat.

The purpose of the present study was to 1) determine the subcellular distribution of enzymatic activities toward PNG in rat intestinal mucosa as a function of vitamin B-6 status and, 2) examine the differences between AIN-76A and AIN-93G purified diets on PNG hydrolase activity in the cytosolic and membrane-associated subcellular compartments.

## Materials and Methods

**Animals and diet**. Weanling male rats (Hsd:Sprague Dawley SD; Harlan Laboratories, Indianapolis, IN), weighing ~50 g were used in both study 1 and 2. Rats were housed in hanging wire mesh stainless steel cages and maintained at constant temperature with 12h light:dark cycle. All procedures for animal care and treatment were approved by the Institutional Animal Care and Use Committee at the University of Florida.

In study 1, the rats (n=29) were randomly assigned to one of four AIN-93G purified diets (107) from Dyets, Inc. (Bethlehem, PA) that differed only in the concentration of pyridoxine. Rats were given free access to diets that provided 7 mg/kg (n=5), 1 mg/kg (n=6), 0.5 mg/kg (n=6), and 0 mg/kg (n=6) pyridoxine-HCl. Another group of rats (n=6) was pair-fed a diet containing 7 mg/kg pyridoxine (7 mg/kg, pf) matched to the intake of rats fed the 0 mg/kg diet. Animal weight and food intake were monitored daily over the study period. In study 2, rats (n=49) were fed one of 10 AIN-formulated purified diets (107) from Dyets, Inc. (Bethlehem, PA). Rats were randomly assigned to either basal diet AIN-76A or AIN-93G: AIN-76A with 2 mg/kg (n=5), 1 mg/kg (n=4), 0.5 mg/kg (n=5), 0.1 mg/kg (n=5), or 0 mg/kg (n=5) PN-HCl, or AIN-93G with 2 mg/kg (n=5), 1 mg/kg (n=5), 0.5 mg/kg (n=5), 0.1 mg/kg (n=5), or 0 mg/kg (n=5) PN-HCl. Study 2 was conducted at a time separate from study 1.

After 5 weeks, the rats in both studies were anesthetized by Halothane (Alocarbon Laboratories, River Edge, NJ) inhalation and exsanguinated by cardiac puncture. Blood was collected with heparinized needles and syringes and transferred to evacuated tubes containing EDTA as an anticoagulant. Plasma was obtained by centrifugation at 2,000 x g for 20 minutes and stored at –80 °C until analysis. Small intestine was harvested, flushed with 9 g/L NaCl at 4 °C to remove intraluminal contents, cut longitudinally, and mucosa was obtained by scraping with a glass slide, all of which were done on ice or at 4 °C.

All procedures were done under gold fluorescent light to minimize photochemical degradation of vitamin B-6.

**HPLC analysis of vitamin B-6 concentrations**. Plasma pyridoxal 5'-phosphate (PLP) was measured as the semicarbazone derivative by reverse-phase fluorometric HPLC using a modification of the method of Ubbink et al. (109). PN concentration of both AIN-76A and AIN-93G diets was measured using reverse-phase fluorometric HPLC (110) after extraction as described previously (4). This analysis was not performed on the diets used in study 1.

**Tissue preparation for enzyme activity assays**. Intestinal mucosa was homogenized using a Potter-Elvehjem tissue homogenizer in 5 volumes of homogenization buffer containing 25 mmol/L sodium phosphate, pH 7.4, containing 50 mmol/L mannitol, 1 mmol/L EDTA, and a general use protease inhibitor cocktail from Sigma Chemical Co. (St. Louis, MO). The cytosolic and total membrane fractions were obtained by centrifugation of the mucosal crude homogenate at 200,000 x g for 30 min at 4°C. Cytosolic supernatant was removed and the resulting membrane pellet was resuspended in homogenization buffer using a Potter-Elvehjem homogenizer. Brush border membrane was isolated according to the method of Kessler et al. (111) with some modification (112). Briefly, non-brush border membranes were precipitated by the addition of solid $MgCl_2$ (to yield a final concentration of 10 mmol/L) to a portion of mucosal crude homogenate, followed by centrifugation at 3,000 x g for 10 min. The supernatant was centrifuged at 40,000 x g for 20 min to obtain a brush border pellet. The pellet was resuspended using a Potter-Elvehjem homogenizer and centrifuged again at 40,000 x g for 20 min. The final brush border membrane pellet was resuspended in homogenization buffer with a Potter-Elvehjem homogenizer. Using the specific activity of the brush border membrane enzyme sucrase, brush border membrane isolation consistently yielded an enrichment factor of ~10-14, which is consistent with other published values (111,113). Using PNG as the substrate, the crude homogenate and cytosolic, total membrane, and brush border membrane subcellular fractions of intestinal mucosa were assayed for hydrolytic activity in study 1 and cytosolic and total membrane subcellular fractions were assayed for activity in study 2.

**Measurement of PNG hydrolytic activity.** *In vitro* assays of enzyme activity were done on the different subcellular fractions of intestinal mucosa using PNG as the substrate. PNG was prepared by biological synthesis using alfalfa seeds and purified as described by Gregory and Nakano (110). All activity assays were done under conditions that allowed the measurement of initial rate.

The assay for the hydrolysis of PNG was done by a minor modification of the method of Nakano and Gregory (13). Reaction mixtures contained 80 mmol/L sodium phosphate, pH 6.0, and 0.25 mmol/L PNG. Reaction mixtures were incubated for 60 min at 37 °C and terminated by incubation in 100 °C water bath for 5 min. The amount of pyridoxine (PN) released was measured by reverse-phase HPLC with fluorometric detection (13). Protein concentration was determined using a colorimetric assay (114).

**Statistical analyses**. In study 1, differences in plasma PLP and PNG hydrolytic enzyme activities among the rats fed the diets containing different concentrations of pyridoxine were analyzed using one-way analysis of variance (115) and Student-Newman-Keuls pairwise comparison test with SigmaStat software (Jandel Corporation, San Rafael, CA). In study 2, linear regression was used to analyze raw or log transformed data. This was done to account for small differences in measured PN concentration in the AIN-76A and AIN-93G diets. Rat weight gain, plasma and liver PLP concentrations, and enzymatic activities were analyzed by a t-test comparison of linear regression line slopes and y-intercepts between the AIN-76A and AIN-93G diet groups (116) using Prism software (GraphPad Software, Inc., San Diego, CA). Plasma PLP concentration was log transformed to normalize the distribution and variance. Dietary PN concentrations were log transformed to linearize the liver PLP concentrations. A P value of less than 0.05 was considered to be statistically significant. Data are presented as means ± SEM.

## Results

**Diet and nutritional status**. In study 1, rat growth significantly increased ($P<0.0001$) as dietary PN concentration increased, with final body weights of 255 ± 4 g (7 mg/kg, ad libitum), 197 ± 5 g (7 mg/kg, pair-fed, pf), 229 ± 3 g (1 mg/kg), 222 ± 6 g (0.5 mg/kg), and 164 ± 4 g (0 mg/kg). The AIN-76A and AIN-93G diets from study 2 were analyzed for pyridoxine (PN) concentrations at the conclusion of the study. At each intended level of fortification, pyridoxine concentrations were consistently higher in the AIN-76A diets than in the AIN-93G diets. The PN content of AIN-76A and AIN-93G diets (in parentheses) was: AIN-76A; 2 mg/kg PN (2.2 mg/kg), 1 mg/kg (1.1 mg/kg) 0.5 mg/kg (0.52 mg/kg), 0.1 mg/kg (0.12 mg/kg), 0 mg/kg (0.05 mg/kg), and AIN-93G; 2 mg/kg (1.8 mg/kg), 1 mg/kg (0.88 mg/kg), 0.5 mg/kg (0.42), 0.1 mg/kg (0.09 mg/kg), 0 mg/kg (0.03 mg/kg). In study 2, weight gain (final weight (g) – initial weight (g)) significantly increased with increasing concentrations of dietary PN in rats fed either AIN-76A or -93G diets (P<0.0001). The regression lines describing the weight gain of rats fed AIN-76A or AIN-93G as a function of dietary PN did not have significantly different slopes (Figure 2-1), but did exhibit different y-intercepts. The y-intercept of the regression line describing weight gain was significantly higher (P<0.001) for rats fed AIN-93G than the y-intercept of the regression line of the rats fed the AIN-76A diet (153.5 vs. 135.7 g).

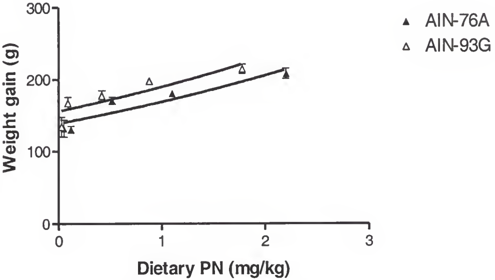


Figure 2-1. Linear regression analyses of weight gain of rats fed AIN-76A and AIN-93G with different concentrations of pyridoxine (PN) after 5 wk. The slopes of the lines were not significantly different (P= 0.7322); pooled slope=36.4. The y-intercepts of the regression lines were significantly different (P<0.001).

In study 1, increases in dietary PN concentration improved vitamin B-6 nutritional status among the different groups of rats. Concentrations of PLP in liver and plasma in study 1 were normally distributed with equal variance. PLP concentrations in plasma and liver significantly increased (P<0.0001) with increasing dietary PN (Table 2-1). In study 2, plasma PLP concentrations were log transformed to meet the assumptions of normal distribution and equal variance. Plasma and liver PLP concentrations for rats fed

Table 2-1. Indicators of vitamin B-6 nutritional status and mucosal hydrolytic activity toward PNG in the small intestine of rats after consuming pyridoxine defined diets for 5 wk.

| | **Vitamin B-6 Nutritional Status**[1] | | **PNG hydrolytic activity** | |
|---|---|---|---|---|
| **Dietary Vitamin B-6** (mg PN/kg diet)[2] | **Plasma PLP** (nmol/L) | **Liver PLP** (nmol/g) | **Cytosol** (nmol PN/h*mg protein) | **Brush border membrane** (nmol PN/h*mg protein) |
| 7 mg/kg | $1352 \pm 59^{a}$ | $16.3 \pm 0.3^{a}$ | $1.1 \pm 0.3^{a}$ | $11.2 \pm 0.6^{a}$ |
| 7 mg/kg pf | $726 \pm 92^{a}$ | $15.6 \pm 1.5^{a}$ | $0.7 \pm 0.2^{a}$ | $20.7 \pm 1.2^{b}$ |
| 1 mg/kg | $229 \pm 34^{b}$ | $13.0 \pm 1.2^{ab}$ | $0.5 \pm 0.2^{a}$ | $17.8 \pm 2.1^{ab}$ |
| 0.5 mg/kg | $165 \pm 9^{b}$ | $9.7 \pm 1.2^{b}$ | $0.4 \pm 0.2^{a}$ | $16.7 \pm 0.7^{ab}$ |
| 0 mg/kg | $45 \pm 12^{bc}$ | $9.2 \pm 1.1^{b}$ | $0.9 \pm 0.4^{a}$ | $13.2 \pm 1.4^{a}$ |

[1] Values are means ± SEM. Means within a column with unlike letters were found to be statistically different at $P<0.05$.

[2] Abbreviations used: PNG, pyridoxine glucoside; PN, pyridoxine; pf, pair fed; PLP, pyridoxal-5'-phosphate

both AIN-76A and AIN-93G purified diets significantly increased ($P<0.001$) in response to increasing concentrations of PN in the diets. Plasma PLP concentrations of the rats fed the AIN-76A or AIN-93G diet increased similarly with increasing dietary PN concentration as the slopes of the lines were found to not be statistically different ($P=0.54$). Similarly, the y-intercepts of the regression lines for plasma PLP concentrations were also not statistically different ($P=0.75$). Liver PLP concentrations were not statistically different between the groups fed the two basal diets as interpreted from the parallel slopes of the regression lines ($P=0.6793$). The y-intercept for the regression line for liver PLP concentrations of the rats fed AIN-76A was significantly higher ($P=0.009$) than the y-intercept for rats fed the AIN-93G diet (Figure 2-2).

**PNG hydrolytic activities.** In study 1, PNG hydrolytic specific activities of the small intestinal mucosa were determined in the cytosolic and brush border membrane subcellular fractions using PNG as the substrate (Table 2-1). The activity measured in the brush border membrane fraction was significantly greater in rats that were pair-fed the 7 mg/kg PN than rats fed the 7 mg/kg and 0 mg/kg diets (ad libitum). There was no effect of dietary PN concentration or pair-feeding on PNG hydrolytic specific activity measured in the cytosolic fraction (P=0.35).

Enzymatic activity data from study 1 were used to make further comparisons among the intestinal mucosa subcellular fractions (crude homogenate, cytosol, total and brush border membrane) within the different dietary PN concentrations (Figure 2-3). With PNG hydrolytic activity expressed on the basis of mucosal weight, the subcellular distribution of activity could be assessed. Although dietary PN concentration did not affect the subcellular distribution of PNG hydrolysis, the activity measured in the total and brush border membrane fractions was significantly greater than that measured in cytosolic fractions (P<0.001). Recovery of brush border membrane from the crude homogenate was ~45% as measured by lactase activity. After adjusting PNG hydrolytic activity for its respective recovery, brush border membrane accounted for 50-60% of the total PNG hydrolytic activity measured in intestinal mucosa, while cytosolic activity contributed 10% of the total PNG hydrolytic activity.

In study 2, PNG hydrolytic activity in the cytosolic fraction significantly increased with increasing dietary PN in rats fed the AIN-76A (P=0.02), but not the AIN-93G diet (P=0.6547). PNG hydrolytic activity measured in the total membrane fractions was not influenced by dietary PN in rats fed either basal diet (regression line slopes that were not significantly different from zero). The slopes of the regression lines describing the PNG hydrolytic activity in the cytosolic and total membrane fractions were not significantly different between groups fed the AIN-76A or AIN-93G diets (Figure 2-4). However, rats fed the AIN-93G diet had greater hydrolytic activity toward PNG in the cytosolic (P<0.0001) and total membrane (P=0.006) fractions than rats fed the AIN-76A diet,

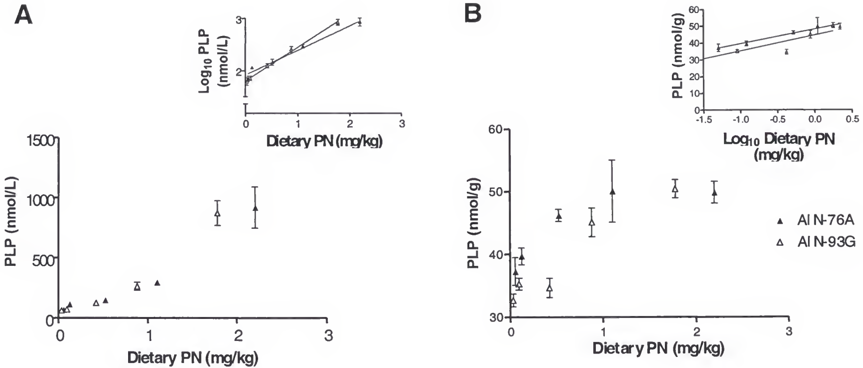


Figure 2-2. Linear regression analyses of pyridoxal-5'-phosphate (PLP) concentrations in rats fed AIN-76A and AIN-93G. (A) Plasma PLP concentrations were log transformed (inset) for analysis. Regression lines for plasma PLP concentrations did not have significantly different slope (P=0.5397), or y-intercept (P=0.7453). (B) Dietary PN concentrations were log transformed (inset) for analysis. Regression lines for liver PLP concentrations did not have different slopes (P=0.6793), but significantly different y-intercepts (P=0.009).

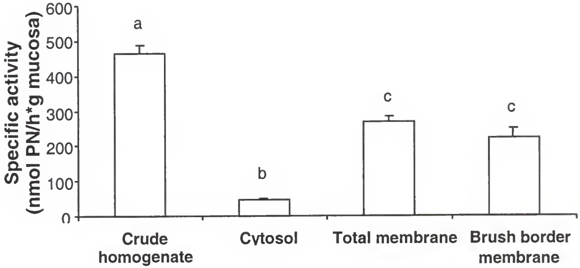


Figure 2-3. Subcellular distribution of PNG hydrolytic activity (nmol PN/h*g mucosa) in the small intestinal mucosa from rats fed AIN-93G with different concentrations of PN. Subcellular fractions tested included: crude homogenate, cytosol, total membrane, and brush border membrane. Bars with different letters within a dietary concentration of PN indicate significance at $P<0.05$.

irrespective of dietary PN concentration. This was concluded from the parallel slopes, but significantly different y-intercepts of the regression lines describing these data. When PNG was used as a substrate, there was a trend toward higher specific activities in the total membrane fraction than in the cytosolic fraction, regardless of diet formulation (AIN-76A or AIN-93G) or PN concentration, which is consistent with the data in study 1.

## Discussion

The results reported here extend our understanding of the bioavailability of vitamin B-6 with focus on the partial hydrolysis of PNG. We were particularly interested in the subcellular localization of PNG hydrolysis and the effect of vitamin B-6 nutritional status on the hydrolytic enzyme activities toward PNG in the rat small intestine.

We observed pronounced changes in plasma and liver PLP concentrations in response to variations in dietary PN concentration in both studies that were in general agreement with other studies of rats fed PN defined diets (6,117-119). Both AIN-76A

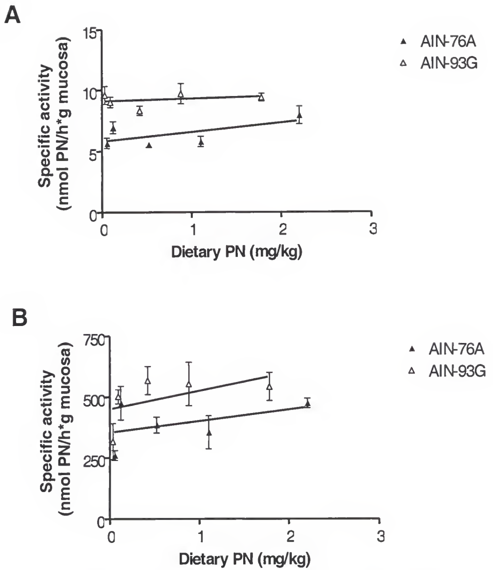


Figure 2-4. Regression analyses for PNG hydrolytic activity (nmol PN/h*g mucosa) in rats fed AIN-76A and AIN-93G. (A) Regression lines for PNG hydrolytic activity in the cytosolic fraction did not have significantly different slope (P=0.2368), but did exhibit significantly different y-intercepts (P<0.001). (B) Regression lines for PNG hydrolytic activity in the total membrane fraction did not have significantly different slope (P=0.637), but did exhibit significantly different y-intercepts (P=0.007).

and AIN-93G diets yielded increased plasma and liver PLP concentrations as dietary PN increased. Liver, but not plasma, PLP concentrations appeared to be influenced by another dietary component in addition to PN since the y-intercept of the regression line for the AIN-76A diet was significantly higher than the AIN-93G diet.

The absence of a robust effect of dietary PN on cytosolic PNG hydrolytic activity was contrary to earlier work done in this laboratory which showed an inverse relationship between dietary PN and cytosolic PNG hydrolase specific activity toward PNG in adult rats (13). In that study (13), mucosal cytosolic PNG hydrolytic activity in rats fed a purified diet (AIN-76A) providing 0 mg/kg PN was twice that measured in rats fed 2 mg/kg PN. Similarly, Banks et al. (12) reported the same magnitude of change in mucosal cytosolic PNG hydrolase activity, using PNG as a substrate, in guinea pigs fed diets providing 0 and 3 mg/kg dietary PN. Rats in the present investigation were chronically fed purified diets (either AIN-93G or AIN-76A) providing a wide range of dietary PN concentrations, but exhibited no significant increase in *in vitro* cytosolic PNG hydrolase specific activity with decreasing dietary PN concentration. However, rats that were pair-fed an adequate concentration of PN (7 mg/kg) exhibited a mean PNG hydrolytic activity in the brush border membrane that was twice that measured in rats fed 7 mg/kg PN ad libitum in study 1. This was likely to be a PN-independent effect of food intake that might have altered overall rates of protein synthesis or degradation. The combined data from the two studies indicate a trend for increasing dietary PN concentration to increase PNG hydrolytic activity in not only the cytosolic fraction, but also the brush border membrane subcellular fraction of intestinal mucosa.

Basal diet composition was speculated to have an effect on the vitamin B-6 nutritional status and possibly intestinal enzymatic activities, which led to the comparison of AIN-76A and AIN-93G purified diets. The predominantly starch-based carbohydrate component of AIN-93G may be a more favorable energy source for metabolically active microflora that synthesize vitamin B-6, assuming that some of the starch is not fully hydrolyzed in the rat small intestine. Under these conditions, the microflora could make more vitamin B-6 available for absorption, and further, improve the vitamin B-6 nutritional status of a rat not receiving an adequate amount of dietary vitamin B-6. Rats fed the

AIN-93G diets had a significantly higher final body weight gain than rats fed AIN-76A, regardless of PN concentration. This suggested that there might be an effect of basal diet composition on the nutritional status of growing rats. A recent comparison of the AIN-76A and AIN-93G diets found only gastric hisotpathological differences in rats fed the purified diets, with more pathologies occurring in rats fed the AIN-76A diet (120). No weight differences between rats fed either the AIN-93G or AIN-76A diets were detected; however, rats examined by Lien et al. (120) were at least 3 times larger and more mature than the weaning rats used in our study. There is a possibility that rapidly growing (weaning) rats respond differently to the two basal diet formulations.

Results from study 2 also indicated that changes in mucosal PNG hydrolytic activity in the rat small intestine are influenced by basal diet composition and not only dietary PN. Rats fed the AIN-93G diet had significantly greater cytosolic and total membrane hydrolytic activity than rats fed AIN-76A, regardless of PN concentration. However, rats in study 2 that were fed AIN-76A exhibited a positive relationship between cytosolic PNG hydrolase activity and dietary PN concentration. In contrast, this effect was absent in the rats fed the AIN-93G. These results indicate that the *in vitro* hydrolysis of PNG by rat intestinal mucosa is actually increased with increasing dietary PN concentration and may be further influenced by other dietary components that affect the small intestine in general (e.g. protein synthesis, processing, or degradation).

In the present study, we were interested in determining the subcellular distribution of PNG hydrolytic activity in intestinal mucosa and potential effects of diet composition on this distribution. Our laboratory has recently discovered that the intestinal brush border membrane hydrolysis of PNG is catalyzed by LPH (106), which complements the intracellular hydrolysis of cytosolic PNG hydrolase reported previously (14). To make direct comparisons of PNG hydrolytic activities among subcellular fractions obtained from intestinal mucosa, *in vitro* activity from study 1 was expressed relative to mucosal weight. The greatest PNG hydrolytic activity was detected in the crude homogenate, which is comprised of all subcellular fractions. Activity measured in the total and brush border membrane fractions was greater than the activity measured in the cytosolic regardless of dietary PN concentration. Since our isolation of brush border membrane was incomplete, we adjusted the PNG hydrolytic activity in the brush border membrane to reflect 100% recovery. We found that 50-60% hydrolytic activity toward PNG in the intestinal mucosa was localized to the brush border membrane of rat intestinal mucosa. Similarly, we found that nearly all of the activity measured in the total membrane fraction could be attributed to the brush border membrane. Although the primary function of LPH is to catalyze the hydrolysis of dietary lactose, this laboratory has found it to hydrolyze PNG, and further evidence of secondary substrates for LPH has been recently reported (121).

The results from the present study, along with our recent kinetic analysis of LPH-catalyzed PNG hydrolysis (106) and partial characterization of the cytosolic PNG hydrolase (14), we have constructed a model to explain the intestinal absorption and processing of dietary PNG. A fraction of the PNG that enters the intestinal lumen may not be absorbed at all and, consequently, may be excreted in the feces. PNG also may be absorbed as the intact glucoside and excreted in the urine unchanged or undergo intracellular hydrolysis by cytosolic PNG hydrolase, which would release free PN. Alternatively, PNG could be hydrolyzed at the brush border membrane by LPH and absorbed as free PN and glucose. In addition to the partial hydrolysis of PNG that occurs in the intestine, there is evidence of hydrolysis in other organs. An investigation of PNG bioavailability in humans, using stable isotopically labeled [$^2$H]-PNG, reported the recovery of the label in the vitamin B-6 urinary metabolite, 4-PA, from an intravenous dose, which suggested that some PNG was hydrolyzed outside of the intestine (10). These data are further supported by the observations of Nakano and Gregory (13). *In* *vitro* assays of PNG hydrolytic activity found that rat kidney, along with the intestinal mucosa, was able to partially hydrolyze PNG. Overall, these data have advanced our understanding of the absorption and metabolism of PNG.

## CHAPTER 3

## ENZYMATIC HYDROLYSIS OF PYRIDOXINE-5'-β-D-GLUCOSIDE IS CATALYZED BY INTESTINAL LACTASE-PHLORIZIN HYDROLASE

### Introduction

A significant dietary source of vitamin B-6 for humans is provided by a glycosylated form of the vitamin, pyridoxine-5'-β-D-glucoside (PNG). PNG, found only in foods of plant origin, provides a mean of 15% of total vitamin B-6 in a mixed diet, depending on food selection (30,64). PNG exhibits an approximate 50% bioavailability in humans (10,11) and 25-30% in rats (6-9) relative to pyridoxine, the metabolically usable form of vitamin B-6. The rate-limiting step in the utilization of PNG is not its intestinal absorption, but rather the enzymatic hydrolysis of the β-glucosidic linkage to release glucose and pyridoxine in the intestine (6-8). Although PNG can be absorbed intact, this form is not metabolized or retained by the liver (6-8) and can antagonize the metabolism of nonglycosylated forms of vitamin B-6 (5,63). The remainder of PNG that is not absorbed is likely to be accounted for through fecal losses.

This laboratory has examined the intracellular, i.e. cytosolic, hydrolysis of PNG in mammalian small intestinal mucosa. Intestinal cytosolic PNG hydrolysis was initially thought to be catalyzed by a broad specificity β-glucosidase (BSβG) (EC 3.2.1.21) (66), a cytosolic enzyme found in the intestine and liver (12,13,104). Cytosolic PNG hydrolysis was also reported to be inversely related to vitamin B-6 nutritional status in rodents (12,13). To further study cytosolic PNG hydrolysis, this laboratory purified BSβG from pig intestinal mucosa, which led to the identification and subsequent purification of a distinct and novel cytosolic β-glucosidase, designated pyridoxine-5'-β-D-glucoside hydrolase (PNG hydrolase)(14). It was found that PNG hydrolase, not BSβG, was responsible for the cytosolic cleavage of PNG. Partial characterization of cytosolic PNG hydrolase revealed its ability to hydrolyze PNG as well as lactose and cellobiose, but not sucrose (14). Partial amino acid analysis revealed substantial sequence homology, but significant differences between PNG hydrolase and the brush border membrane enzyme lactase phlorizin hydrolase (LPH) (EC 3.2.1.23, 62, and 108)(74,122), the enzyme primarily responsible for the hydrolysis of dietary lactose (C.W. Tseung, L.G. McMahon, and J. F. Gregory, unpublished).

A recent reassessment of mucosal PNG hydrolase activity in the rat small intestine indicated that ~50% of the activity associated with the total membrane fraction could be attributed to enzymatic activity in the brush border membrane (A. D. Mackey, et al., unpublished). Hydrolysis of glucosides and oligosaccharides by enzymes present in the intestinal brush border often precedes the absorption of individual monosaccharides or the aglycone of glycosylated molecules, such as flavonoid and isoflavone glucosides. Day and colleagues (121) found that quercetin, genistein, and daidzen β-glucosides were hydrolyzed by purified sheep LPH prior to absorption. LPH is the only mammalian brush border β-glucosidase; thus, we hypothesize that LPH is responsible for the hydrolysis of PNG, a β-glucoside. LPH has two distinct catalytic active sites: one for the hydrolysis of lactose and flavonoid glucosides and another, phlorizin hydrolase, for the hydrolysis of phlorizin and β-glucosylceramides (75,123). As reported by Day et al. (121), the site of catalysis for flavonoid and isoflavonoid glucoside hydrolysis was experimentally assigned to the lactase active site, not the phlorizin hydrolase site.

The kinetic characterization of LPH-catalyzed hydrolysis of PNG by purified rat LPH is reported herein including the use of lactose as a substrate for comparison of catalytic properties. We also report the *in vitro* formation of a PN-disaccharide, which is evidence of a transferase activity for mammalian LPH.

## Materials and Methods

**Materials.** Pyridoxine (PN) hydrochloride, lactose, glucose, phloridizin (phlorizin), phloretin, Sephacryl S400, Sephacryl S200, 2',4'-dinitrophenyl-2-fluoro-2-deoxy-β-D-glucopyranoside (2F-DNPGlc), D-glucal, protease inhibitor cocktail, goat-anti-rabbit IgG horseradish peroxidase antibody, and a glucose detection kit were obtained from Sigma Chemical Co. (St. Louis, MO). Prestained protein molecular mass markers were obtained from Invitrogen (Carlsbad, CA). DEAE cellulose was obtained from Whatman (Clifton, NJ), and Simply Blue SafeStain was purchased from Invitrogen (Carlsbad, CA). Maleimide-activated keyhole limpet hemocyanin (KLH) was obtained from Pierce (Rockford, IL). Polyvinyldifluoride (PVDF; Immobilon-P) was obtained from Millipore Corporation (Bedford, MA). ECL Plus chemiluminescent reagent was purchased from Amersham-Pharmacia Biotech (Buckinghamshire, England). Pyridoxine-5'-β-D-glucoside was prepared by biological synthesis from pyridoxine using germinating alfalfa seeds and purified chromatographically (13,110).

**Purification of lactase phlorizin hydrolase (LPH).** LPH was purified according to the Triton X-100 solubilization method of Wacker et al. (75) with the exceptions of Sephacryl S200 substituted for Sephadex G-200 and omission of the final anti-lactase affinity purification column. During this purification, all materials were kept on ice or at 4 °C. Brush border membrane was isolated from rat intestine for the purification of LPH according to the method Kessler et al. (111) with some modifications (112). Weanling male and female rats (Hsd:Sprague-Dawley, Harlan, Indianapolis, IN) (n=45) were used for the purification of LPH. Small intestine was harvested, flushed with 0.9% (w/v) NaCl, cut longitudinally, and mucosa was scraped with a glass slide, yielding approximately 20 g of total mucosa. Electrophoretic characterization of the purified enzyme was done under denaturing conditions using 8% (w/v) polyacrylamide gels according to Laemmli (124) using a mini-gel electrophoresis apparatus from Novex (Invitrogen; Carlsbad, CA). A commercially available prestained protein molecular mass standard ranging from 10-173 kD was used. Gels were stained with Simply Blue SafeStain for 1 h followed by destaining overnight in distilled, deionized water.

**Antibody production and Western blot analysis**. The peptide CTLFHFDLPQALEDQG was synthesized with an additional cysteine at the carboxyl-terminus and verified by mass spectrometry amino acid analysis by Research Genetics (Huntsville, AL). This peptide was conjugated at the terminally added cysteine residue to maleimide-activated keyhole limpet hemocyanin (KLH) according to manufacturer instructions. KLH-conjugated peptide was injected into New Zealand White Rabbits (Cocalico Biologicals, Reamstown, PA) for production of polyclonal antiserum. Immunoblotting, or Western analyses, were done as described by Harlow and Lane (125). Purified LPH protein was resolved on an 8% (w/v) SDS-PAGE at 130 V for 2 h. The gel was electroblotted to polyvinyldifluoride (PVDF) for 2.5 h at 12V in a transfer buffer containing 192 mmol/L glycine, 25 mmol/L Tris/ 0.05% SDS/10% methanol. The blot was stained with Amido black and destained. For the protein detection, the stained blot was cut into strips for different antisera treatments. Primary antibody treatments included whole immune serum, pre-immune serum, and peptide-competed serum. The peptide-competed serum was prepared by incubating the whole immune serum (0.5 mL) with 10 μg of peptide at room temperature for 1 h prior to application to the blot. The strips were blocked in phosphate buffered saline (PBST) (137 mmol/L NaCl/2.7 mmol/L KCl/5.4 mmol/L $Na_2HPO_4$/1.8 mmol/L $KH_2PO_4$/0.05% (v/v) Tween-20) containing 5% (w/v) nonfat dry milk (NFDM) for 1 h. Primary antibody treatments were applied at 1:500 dilution (in PBST-NFDM) followed by washing with PBST without NFDM and subsequent addition of the secondary antibody (goat anti-rabbit IgG horseradish peroxidase conjugate) at 1:5000 dilution. Unbound secondary antibody was removed by washing with PBST (no NFDM). A chemiluminescent/fluorescent substrate (ECL-Plus) was then applied and incubated at room temperature for 5 min. The excess substrate was blotted away and protein bands were visualized by fluorescence emission (Storm 840 fluorescent optical scanner; Amersham-Pharmacia Biotech; Sunnyvale, CA).

**Enzyme activity assays**. The standard assay for PNG hydrolytic activity was performed according to Nakano and Gregory (13). Standard activity assays for PNG hydrolysis were performed in a reaction mixture containing 10 µg purified LPH, 0.25 mmol/L PNG in 80 mmol/L sodium phosphate, pH 6.0 that was incubated at 37 °C for 1 h. Pyridoxine release was measured using reverse-phase HPLC with fluorometric detection (110). Lactase activity was measured according to Dahlqvist (96) with modification of the 50 mmol/L maleate buffer to a 50 mmol/L sodium phosphate, pH 6.0 assay buffer. Standard activity assays for lactose and other disaccharide hydrolysis were done using 25 mmol/L disaccharide. Glucose release was quantified using a reagent kit based on the glucose oxidase reaction with colorimetric detection. Phlorizin hydrolase activity was measured by reverse phase HPLC with UV detection of the aglycone, phloretin (126). Standard assay mixtures contained 100 mmol/L phlorizin in 50 mmol/L sodium citrate, pH 5.9. All activity assays were conducted under conditions that allowed measurement of initial rate.

For kinetic studies, reactions involving LPH-catalyzed hydrolysis of PNG and lactose were conducted at various concentrations as specified in text or tables. Experiments examining active site inhibition, along with selective active site protection using 2F-DNPGlc and a glycal, D-glucal, were conducted as described by Day et al. (121) and Arribas et al. (123). Other potential inhibitors as specified were added to standard reaction mixtures without pre-incubation of enzyme. Kinetic constants ($K_m$,

$V_{max}$, and $K_i$) were calculated by nonlinear regression using SigmaPlot Enzyme Kinetics Module, v 1.0 (SPSS, Inc., San Rafael, CA) software.

Protein concentration was determined by a colorimetric method (114) using bovine serum albumin as the standard.

**Liquid chromatography-mass spectrometry (LC-MS).** To obtain molecular mass and fragmentation information regarding a novel reaction product formed during incubation of purified LPH with PNG and lactose, LC-MS and LC-MS/MS analyses were performed in atmospheric pressure chemical ionization (APCI) mode (LC: Hewlett Packard model 1100; Agilent; Palo Alto, CA and MS: Thermo Finnigan model TSQ7000; San Jose, CA). The HPLC separation was done isocratically with 5 mmol/L ammonium acetate with 0.1% (v/v) acetic acid as the mobile phase at a flow rate of 0.6 mL/min with a reverse-phase column (C18 Phenosphere, 50 x 4.6 mm, 5 µm column; Phenomenex; Torrance, CA). The analysis was conducted by monitoring ions from 75-600 m/z. For the MS-MS analysis, a collision energy of 30 V was used to fragment the parent mass (M+1) of m/e 494.

## Results

**Lactase phlorizin hydrolase (LPH) purification.** LPH from rat small intestinal mucosa was purified 300-fold relative to the lactase activity measured in mucosal crude homogenate. The enzyme was purified to near homogeneity and the purification repeated with similar results. The approximate molecular mass of purified LPH by SDS-polyacrylamide gel electrophoresis was 135-140 kDa. In the published method used for purification, an anti-lactase antibody affinity column was used to further separate LPH from aminopeptidase N (MW 145 kD) (75). This investigation did not use an affinity chromatography purification step to remove peptidase activity. Although aminopeptidase N may not have been completely separated from the final purified LPH, the enrichment of lactase activity from the crude homogenate was consistent with that reported in the purification method (75). Staining of the SDS-PAGE revealed two major bands of protein: one at ~135-140 kD and another at ~200-220 kD (Figure 3-1A). The band of protein at 135-140 kD corresponds to mature LPH. The band at 200-220 kD is consistent with the MW of a precursor form of LPH (91). Western analyses were done to confirm further the relative purity of the enzyme preparation (Figure 3-1B). The peptide used for antibody production had amino acid sequence homology with regions in the precursor and mature forms of LPH, but no other mammalian intestinal enzymes cataloged in Swiss-Prot and GenBank databases. No protein bands were detected in the blot strips incubated with PBST-NFDM alone and the secondary antibody alone (lanes 1 and 2, respectively). The whole immune serum-treated strip revealed three immunogenic protein bands (lane 3), which were not visible in the peptide-competed antibody treatment (lane 4). The fourth visible band (~60 kD) was found to be an artifact of the antibody under the conditions of this analysis. The band at ~80 kD detected by the whole immune serum was also detected by the pre-immune serum, which indicates that the recognition of the band by the whole immune serum was not sequence specific (lane 5). The protein bands at 135-140 kD and 220-220 kD that were recognized by this polyclonal antibody appeared to be the mature and precursor forms of LPH. Upon closer inspection of the Coomassie-stained gel, trace amounts of additional high molecular mass bands were observed. Further assays for disaccharidase activity of the purified LPH preparation revealed residual maltase-glucoamylase (EC 3.2.1.20 and 3) at a specific activity of 3.8 µmol/min*mg protein. This activity was controlled for in later kinetic characterization studies.

**Kinetic characterization.** Purified LPH from rat small intestinal mucosa catalyzed the hydrolysis of PNG and followed Michaelis-Menten kinetics. Values for $K_m$, $V_{max}$, and $k_{cat}$ were lower when PNG was used as the substrate as compared to lactose. Values for $k_{cat}$ and $V_{max}/K_m$ indicated that lactose hydrolysis was catalyzed more efficiently than the hydrolysis of PNG (Table 3-1).

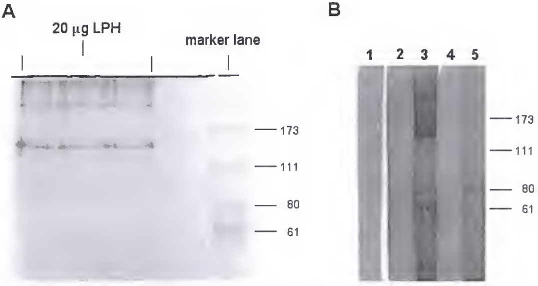


Figure 3-1. Purification of LPH from rat intestine. (A) SDS-PAGE of purified LPH preparation. Gel (8% w/v acrylamide) has two lanes: one large lane containing enzyme preparation and another for the molecular mass marker. Purified enzyme (20 µg) was loaded into one large well. Enzyme preparation was electrophoresed at 130 V for 2 hours. Gel was stained with commercially available formulation of Coomassie blue stain (SimplyBlue SafeStain, Invitrogen). Molecular weight markers ranged from 10-173 kDa. Protein band at 135-140 kDa corresponds to LPH. (B) Western analysis of purified LPH preparation. Protein (20 µg) was separated by SDS-PAGE (8% w/v acrylamide) and transferred to PVDF membrane. Membrane was cut into strips and subjected to various antibody treatments. Lane 1: No antibody applied; Lane 2. Secondary antibody (goat-anti-rabbit HRP) only; Lane 3: Whole immune serum and secondary antibody; Lane 4: Peptide-competed serum and secondary antibody; Lane 5: Pre-immune sera and secondary antibody. Application of chemiluminescent/fluorescent substrate reagent (ECL Plus) was followed by detection using a fluorescent optical scanner.

Table 3-1. Kinetic parameters of purified rat lactase phlorizin hydrolase (LPH) using pyridoxine-5'-β-D-glucoside (PNG) and lactose as substrates.

| | **Substrate** | |
|---|---|---|
| | **PNG** | **Lactose** |
| **$K_m$** (mmol/L) | 1.0 ± 0.1 | 16 ±1 |
| **$V_{max}$** (μmol/min*mg LPH) | 0.11 ± 0.01 | 5.0 ± 0.1 |
| **$k_{cat}$** ($s^{-1}$) | 1.0 | 47 |
| **$k_{cat}/K_m$** ($mmol/L^{-1}s^{-1}$) | 1.0 | 2.9 |
| **$V_{max}/K_m$** | 0.11 | 0.31 |

**Inhibition studies**. The substrate PNG exhibited substrate inhibition at concentrations greater than 3 mmol/L (Figure 3-2). Due to limited availability of PNG, only concentrations ranging from $0.01K_m$ to $5K_m$ were tested. The reaction products (glucose and pyridoxine), lactose, and other disaccharides were also tested for inhibitory properties. Pyridoxine (0.5-10 mmol/L) did not inhibit LPH-catalyzed PNG hydrolysis, but the monosaccharide, glucose inhibited the reaction at concentrations ≥ 25 mmol/L; $K_i$ equal to 64 ± 5 mmol/L. The primary substrate for LPH, lactose (25-100 mmol/L), inhibited PNG hydrolysis competitively with a $K_i$ equal to 56 ± 8 mmol/L (Figure 3-3). This suggests that the two substrates (PNG and lactose) are hydrolyzed at the same active site. Cellobiose, maltose, lactose, sucrose, and trehalose (25-100 mmol/L) were also added individually to standard reaction mixtures containing PNG (0.01-0.25 mmol/L) and purified LPH. While sucrose and trehalose did not affect PNG hydrolysis, cellobiose and maltose inhibited the enzymatic release of PN. The inhibition of PNG hydrolysis by

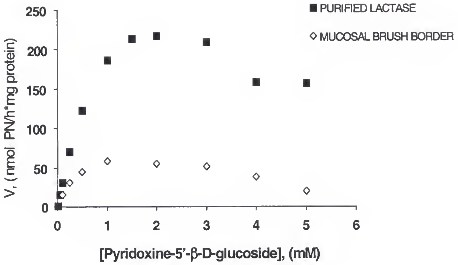


Figure 3-2. Effect of substrate concentration on the activity of LPH-catalyzed PNG hydrolysis. Comparison of rat small intestine brush border membrane and purified LPH is shown.

maltose was reduced by 35-40% in the presence of 2.0 μmol/L 1-deoxynojirimycin, an inhibitor of maltase-glucoamylase activity (127).

**Identification of site of PNG hydrolysis by LPH.** The inhibitor 2F-DNPGlc binds to both the lactase and phlorizin hydrolase active sites, inhibiting both hydrolytic activities (128,129). The lactase activity was preserved by pre-incubation with D-glucal, which yields reversible blockage of the lactase active site (123). Subsequent addition of 2F-DNPGlc to the glucal-modified LPH leads to the selective inhibition of the phlorizin hydrolase active site (123). This experimental protocol allowed the contribution of each activity from the respective active sites to be calculated (123). Hydrolysis of PNG and lactose was reduced 100-fold in the presence of 2F-DNPGlc without glucal as compared to the uninhibited enzyme. Phloretin release was reduced by 85-90% in the presence of 2F-DNPGlc with or without glucal as compared to the activity of the free enzyme. Incubation of purified LPH with glucal prior to the addition of 2F-DNPGlc maintained the hydrolytic activities toward lactose and PNG to 50-65% of the uninhibited enzyme

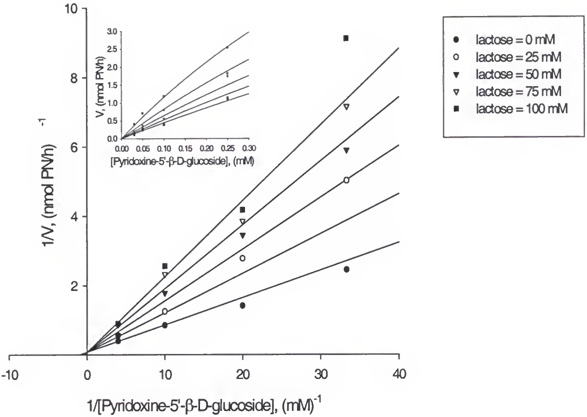


Figure 3-3. Lineweaver-Burk and Michaelis-Menten (inset) plots of LPH-catalyzed hydrolysis of PNG with increasing amounts of lactose as inhibitor.

(Figure 3-4). These findings strongly suggest that PNG was hydrolyzed at the lactase active site of LPH.

**Formation of other pyridoxine glycosides.** HPLC analyses of enzyme reaction mixtures containing LPH, PNG (substrate), and lactose (inhibitor) detected not only the product, PN, and the substrate, PNG, but also another fluorescent compound with a retention time of 5.3 min (Figure 3-5). The amount of this compound formed was dependent on the concentration of lactose. Initial LC-MS (APCI positive ion mode) analysis of the enzyme reaction mixture showed the unidentified compound to have M+1 ion of 494 (m/z), corresponding to a disaccharide of pyridoxine (Fig 3-6). This was

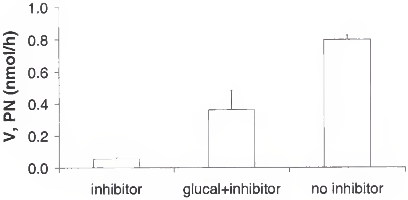


Figure 3-4. Identification of site of PNG hydrolysis by LPH. Protection of active site with D-glucal prior to addition of 2F-DNPGlc (inhibitor) selectively inhibited phlorizin hydrolase active site. Completely inhibited enzyme (with 2F-DNPGlc) exhibited 10% the PNG hydrolytic activity of the glucal-protected enzyme.

further supported by an MS/MS experiment on the M+1 ion using a collision energy of 30V. In addition to the residual M+1 ion of the disaccharide, major fragments were observed at m/z 332 and 170, corresponding to the M+1 ions of pyridoxine glucoside and pyridoxine. The LC-MS and MS/MS experiments produced the same results when the HPLC purified disaccharide from the enzyme reaction mixture was utilized. This pyridoxine disaccharide has a pyridoxine molecule with the 5'-β-D-glucoside and another glucose or galactose moiety. Similar to the observation of the pyridoxine disaccharide formed in the presence of lactose, two other disaccharides tested in the LPH inhibition experiments, cellobiose and maltose, also formed fluorescent compounds which exhibited retention times different from that formed with lactose. These compounds, while detectable, were minor in relation to the amount of the PN-disaccharide formed in the presence of lactose and were independent of disaccharide concentration.

## Discussion

The finding of PNG as a novel substrate for LPH has extended our understanding of mechanisms governing vitamin B-6 bioavailability and has added to the

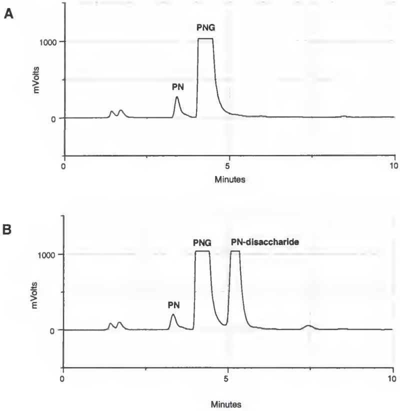


Figure 3-5. Sample chromatograms of enzyme reaction mixtures of LPH-catalyzed PNG hydrolysis. (A), Chromatogram from LPH-catalyzed PNG hydrolysis without added lactose. (B), Chromatogram from LPH-catalyzed PNG hydrolysis with added lactose (25 mM). Retention times: pyridoxine (PN), 3.3 min; pyridoxine-5'-β-D-glucoside (PNG), 4.3 min; pyridoxine disaccharide (PN-disaccharide), 5.3 min. Assays were conducted using purified rat LPH.

growing evidence that LPH catalyzes the hydrolysis of not only lactose, but other β-glucosides, such as plant β-glucans and flavonoid and isoflavonoid glucosides (121,130). Dietary PNG exhibits only 50-60% of the bioavailability of free PN as a source of vitamin B-6 for humans (10,11), which is due to incomplete hydrolysis of the glycosidic linkage of PNG. The results reported in the present study suggest that the digestion of PNG may commence at the intestinal brush border where LPH can catalyze the hydrolysis of PNG releasing free PN, which is then passively absorbed. This extends our previous findings of the hydrolysis of PNG that can occur immediately after

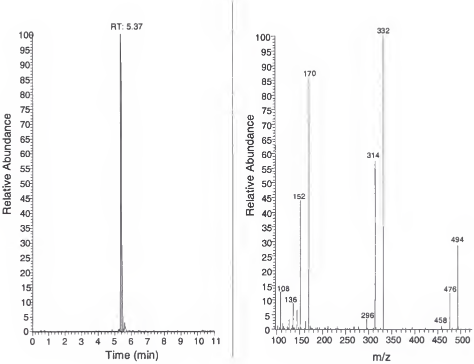


Figure 3-6. LC-MS/MS analysis, in the APCI positive ion mode (MS/MS experiment on the ion at m/e 494; collision energy, 30 V), of the PN-disaccharide product formed during the incubation of LPH with PNG and lactose. The predominant ions are parent molecular ion (M+1) at m/e 332, and M+1 ion of pyridoxine at m/e 170. The other significant ions at m/e 476, 314, and 152 are produced by the loss of water from the respective M+1 ions.

absorption into the cytosolic compartment by PNG hydrolase. This LPH-catalyzed hydrolysis may be an important determinant of the bioavailability of PNG. In populations with lactase deficiency, the hydrolysis, and hence, nutritional utilization of PNG may be less efficient than in populations that exhibit lactase persistence.

LPH was purified from 20d old rats to insure reasonable lactase activity with an adequate amount of starting tissue. The recognition of both the mature and precursor forms of LPH by our antibody in the Western analysis allowed us to verify the qualitative abundance of our purified enzyme. This recognition was sequence specific since the pre-immune serum and peptide-competed antibody did not detect the high molecular mass bands that were recognized by the whole immune serum. Values of $K_m$ and $V_{max}$

(lactose) calculated for purified rat LPH were consistent with published values (73,126)(Table 3-1). The kinetic parameters (for lactose and PNG) appeared to be representative of the concentrations of substrates that would be encountered physiologically by LPH. However, under typical dietary conditions, the amount of PNG in digesta entering the small intestine would be in the micromolar concentration range, while the concentration of lactose (millimolar range) would greatly exceed that of PNG. The relatively high $K_m$ value for PNG (1.0 mmol/L) suggests that LPH has the capacity to hydrolyze physiological concentrations of PNG, but fractional saturation of substrate of the enzyme would be low. Similarly, kinetic ratios also indicated that LPH favored the hydrolysis of lactose over PNG.

Although LPH-catalyzed PNG hydrolysis was inhibited by one of its products, glucose, the concentration at which inhibition occurred may not be physiologically relevant (≥ 25 mmol/L). In accounting for the stoichiometry of the hydrolytic reaction of PNG, the amount of glucose released (micromolar concentration) would not be sufficient to cause product inhibition. However, the glucose released from the hydrolysis of other disaccharides such as lactose, may contribute to the observed inhibition by glucose. The observed competitive inhibition of lactose on LPH-catalyzed PNG hydrolysis is consistent with the fact that PNG and lactose are hydrolyzed at the same active site. The inhibition may not have a dramatic effect on the bioavailability of PNG since foods that contain PNG are of plant origin, which are devoid of lactose. Unless a dairy product and plant-derived food were consumed together, PNG should be at least partially hydrolyzed by LPH. The $K_i$ for lactose (56 ± 8 mmol/L) did not agree with the calculated $K_m$ for lactose (16 ± 1 mmol/L). One possible explanation for this discrepancy may be in the formation of alternative pyridoxine glucosides. Some PNG may be diverted from its β-glucosidic hydrolysis process to a trans-glycosylation reaction that yields a pyridoxine disaccharide and a reduction in free PN. Alternatively, PNG may be hydrolyzed to liberate PN, which may undergo enzymatic modification to form PNG or PN-disaccharide. The mechanism of the hydrolysis/trans-glycosylation was not examined further in this study.

Cellobiose, while not present in appreciable amounts in the human diet, also is a substrate for LPH (74,131); therefore, it was not unexpected that this β-linked disaccharide would also inhibit PNG hydrolysis. The β-disaccharide, maltose also inhibited the release of PN by LPH, which was not anticipated. The subsequent detection of maltase-glucoamylase activity (EC 3.2.1.20 and 3) (80) as a contaminant of the LPH preparation is consistent with the presence of minor high molecular mass protein bands (200-220 kDa) faintly stained in the SDS-PAGE. Western analysis using our antibody that was sequence specific to LPH, but not maltase-glucoamylase, showed that the more prominently stained high molecular mass band was likely to be a precursor form of LPH and not maltase-glucoamylase. Despite the relatively low abundance of the maltase-glucoamylase contaminant, its hydrolytic activity toward maltose had an effect on LPH-catalyzed PNG hydrolysis. What appeared to be the inhibition of PN release, may actually have been the LPH-catalyzed formation of another maltose- or glucose-derived pyridoxine oligosaccharide from PN or PNG, or both. Alternatively, the hydrolysis of maltose releases monosaccharide units (D-glucose) which could inhibit PNG hydrolysis as catalyzed by LPH. Maltase-glucoamylase activity was reduced by 63% in the presence of 2.0 μM 1-deoxynojirimycin without affecting lactose hydrolysis. In reaction mixtures containing LPH, PNG, maltose (≥25 mmol/L), and deoxynojirimycin, the release of PN was increased by an average of 35% and the formation of other pyridoxine disaccharides was decreased compared to reactions without deoxynojirimycin. Increasing the concentration of 1-deoxynojirimycin could have likely negated this inhibition; however, at higher concentrations, deoxynojirimycin also is a competitive inhibitor of lactase (75). Although maltase-glucoamylase was present in the purified LPH preparation, maltase-glucoamylase appears not to alter the kinetics of PNG hydrolysis in the absence of β-linked disaccharides. This is further supported by the inhibition experiment with β-glucosidase inhibitor 2F-DNPGlc. When LPH is completely inhibited with this fluoroglucoside, the release of PN is negligible, indicating that maltase-glucoamylase alone is not capable of hydrolyzing PNG. Maltase-glucoamylase that had been co-purified with lactase by papain solubilization from 15d old rat intestine was reported to have a final specific activity of 36 μmol/min*mg protein in another study (73), which is approximately 10 times the specific activity measured in the current study.

Fluoroglucosides are widely used as general inhibitors of β-glucosidases (129). 2', 4'-dintrophenyl-2-deoxy-2-fluoro-β-D-glucopyranoside (2F-DNPGlc) is a mechanism-based inhibitor of β-glucosidases, and exerts its effect by forming a stable covalent linkage of the 2' position of the fluoroglucoside to glutamic acid residues at the active sites of LPH. The use of glucal in conjunction with the inhibitor in the protocol allows individual examination of the lactase and phlorizin hydrolase active sites on LPH (123). The deglycosylation of flavonoid and isoflavonoid glucosides was studied using this same experimental procedure (121). Inhibition of the phlorizin hydrolase site with 2F-DNPGlc did not diminish the hydrolysis of the quercetin glucosides. Similar to the results reported for the flavonoid and isoflavonoid glucosides (121), we concluded that PNG is hydrolyzed at the lactase active site of LPH.

Various pyridoxine glucosides, including several pyridoxine oligosaccharide derivatives, are known to exist widely in nature (132); however, the formation of pyridoxine disaccharides catalyzed by enzymes in the small intestine has not been reported. Future research will address the nutritional significance of LPH hydrolysis of

PNG, with particular attention to the vitamin in human LPH expression and activity and the inhibition of PNG hydrolysis by lactose.

# CHAPTER 4
# UPTAKE, HYDROLYSIS, AND METABOLISM OF PYRIDOXINE-5'-β -D-GLUCOSIDE IN A CELL CULTURE MODEL USING CACO-2 CELLS

## Introduction

An important source of vitamin B-6 in the human diet is pyridoxine-5'-β-D-glucoside (PNG) which is found in foods of plant origin. This glycosylated form provides approximately 15% of total vitamin B-6 intake in a typical mixed diet; however, this percentage can vary depending on food selection (30). The metabolic utilization of this form of vitamin B-6 is limited by its partial hydrolysis by β-glucosidases in the small intestine. Hydrolysis of PNG yields the products glucose and PN. PN can be metabolized to the coenzyme PLP. Relative to PN, PNG exhibits a 50% bioavailability in humans (10,11) and 25-30% in rats (7,8). The incomplete bioavailability of PNG is due to its limited hydrolysis in the small intestine. This laboratory previously reported the intestinal hydrolysis of PNG to be catalyzed by a novel intracellular β-glucosidase (14). The enzyme specifically hydrolyzed PNG and was later designated cytosolic pyridoxine-5'-β-D-glucoside hydrolase (PNG hydrolase). More recently, this laboratory discovered that the brush border membrane β-glucosidase, lactase phlorizin hydrolase (LPH), also catalyzed the hydrolysis of PNG (106).

LPH is the intestinal enzyme that is responsible for the hydrolysis of dietary lactose, a disaccharide that is important for energy derivation for developing mammals. Although kinetic analyses of purified rat LPH revealed that lactose was a better substrate than PNG, PNG was a secondary substrate for LPH. Further investigation of LPH-catalyzed PNG hydrolysis *in vitro* showed that lactose was a competitive inhibitor of

PNG hydrolysis and therefore, the two substrates are hydrolyzed at the same active site. We speculated that the inhibition by lactose observed *in vitro* would have implications on the *in vivo* hydrolytic and absorptive processes of PNG. Intestinal hydrolysis of PNG *in vivo* might be reduced in the presence of lactose, which would reduce the bioavailability of PNG. Simultaneous consumption of plant-derived foods with dairy products could decrease the hydrolytic release of free PN and consequently, affect the vitamin B-6 nutritional status of the individual. There is also the potential for a reduced PNG bioavailability in lactose intolerant or lactase deficient individuals. Subcellular fractionation of intestinal mucosa revealed that 50-60% of the hydrolysis of PNG was localized to the brush border membrane in the rat small intestine, which was likely catalyzed by LPH (Mackey et al., unpublished). This accounts for a large proportion of the PNG hydrolytic activity present in the small intestine and thus, lactase insufficiency might also reduce PNG bioavailability through the reduction or complete loss of LPH hydrolytic activity toward PNG.

*In vivo* absorption of vitamin B-6 has been extensively examined in the intestine of rats. Intestinal absorption of PN occurs by a non-saturable, passive diffusion process. PN is rapidly absorbed where it is subject to phosphorylation, forming pyridoxine 5'-phosphate (PNP), which can be converted to pyridoxal 5'-phosphate (PLP) by PNP oxidase (133,134). Following the oxidation of PNP to PLP, the intestine dephosphorylates PLP to release PL into portal circulation (134). PNG hydrolysis occurs mostly in the small intestine to release PN, which is passively absorbed; however, PNG, as the glucoside, can be absorbed and excreted intact in the urine (10,13).

Caco-2 human colon carcinoma cells were chosen as a model for the present investigation since these cells, when allowed to differentiate, express the morphology and many of the hydrolytic enzymes present in the intestinal brush border (including LPH) (135), which provides an environment that closely resembles the small intestine.

While these cells express LPH, they were previously found not to exhibit cytosolic PNG hydrolase activity (McMahon, L.G & Gregory, J.F., unpublished data). Hydrolytic activity toward PNG measured in these cells could therefore be attributed solely to the activity of brush border LPH. The purpose of the present study was to examine the uptake and metabolism of PNG in the absence and presence of lactose in a cell culture model using the established Caco-2 human colon carcinoma cell line.

## Materials and Methods

**Materials**. Vitamin B-6 compounds (except PNG), lactose, HEPES, D-glucose, and L-glutamine were purchased from Sigma Chemical Co. (St. Louis, MO). Cell culture grade NaCl, $CaCl_2$, $MgSO_4$, KCl, LiCl, hydroxymethyl aminomethane hydrochloride (Tris-HCl), 2-(-4-morphlolino)-ethanesulfonic acid (MES), and plastic cell culture supplies were purchased from Fisher Scientific (Atlanta, GA). Cell culture media, media supplements, and trypsin-EDTA were purchased from Invitrogen (Carlsbad, CA). PNG was biologically synthesized and purified chromatographically (110).

**Cell culture**. Caco-2 human colon carcinoma cells, passage 18, were obtained from American Type Culture Collection (Rockville, MD). Cells were propagated and maintained at 37°C (95% air, 5% $CO_2$ atmosphere) in DMEM containing 4.5 g/L glucose, 25 mmol/L HEPES, 44 mmol/L sodium bicarbonate, and 4 mmol/L glutamine. The growth medium was supplemented with 1 mmol/L sodium pyruvate, 100 μmol/L non-essential amino acids, and 20% fetal bovine serum plus 100 U/L penicillin, 100 U/L streptomycin, and 50 μg/L gentamycin (136). For routine subculturing, cells were washed with $Ca^{+2}$- and $Mg^{+2}$- free phosphate buffered saline (PBS) and detached with 0.25% (w/v) trypsin with 1 mM EDTA. For uptake experiments, cells were plated on 3-section 100 mm plates at $1.5 \times 10^5$ cells per section. Growth medium was changed every 2-3 days with a change 24 h prior to an uptake experiment. Uptake studies were performed on monolayers 7-9 days post-confluency, a time when lactase activity peaks in Caco-2 cells (137). Cells from passages 23-35 were used in the experiments.

**Uptake experiments.** Uptake experiments were done by adding treatment media containing different concentrations of PNG to the top of the cell monolayer with and without lactose to the cells. Treatment medium was prepared with Krebs-Ringer buffer containing 123 mmol/L NaCl, 4.93 mmol/L KCl, 1.23 mmol/L $MgSO_4$, 0.85 mmol/L $CaCl_2$, 5 mmol/L glucose, 5 mmol/L glutamine, 10 mmol/L HEPES, and 10 mmol/L MES at pH 7.4. Monolayers were washed 3 times with 2-3 mL Krebs-Ringer buffer (37 °C) prior to the addition of treatment media containing different concentrations of PNG and lactose. Treatment media were added to monolayers and incubated at 37 °C in 5% $CO_2$ atmosphere for times as indicated in the text. Prior to the termination of incubation, a sample of treatment medium was collected. Incubation was then terminated at the desired time by addition of 3 mL of Krebs-Ringer buffer (4 °C), followed by two additional washes. Cell monolayers were mechanically lifted from the plates with a sterile plastic spatula into 0.5 mL PBS and homogenized using a Polytron homogenizer at medium speed for 15 sec. An aliquot of crude homogenate was collected for protein measurement and the remaining crude cellular homogenate was centrifuged at 200,000 x g for 30 min to obtain a cytosolic subcellular fraction. Protein concentration was measured spectrophotometrically (114) using bovine serum albumin as the standard.

**Measurement of hydrolysis and uptake.** Ratios of PN:PNG were calculated for the treatment media prior to and after each incubation period. Detection of PNG in the cytosolic compartment of the Caco-2 cells above that measured in cells receiving no additional PNG in the treatment medium was interpreted to be the amount of PNG taken up by the cells.

**Enzyme activity assays.** PNG hydrolytic activity in the cytosolic fraction of Caco-2 cells was determined according to Nakano and Gregory (110) with modification of the assay buffer (106). Lactase activity was measured by a colorimetric assay as described by Dahlqvist (96) with some modification.

**Vitamin B-6 analyses.** Treatment media were analyzed for PN and PNG both before and after incubation period using reverse-phase fluorometric HPLC (110). Intracellular concentrations of PN, PNG, PM, and PMP were measured using ion-pair reverse-phase HPLC with fluorometric detection (4). Intracellular concentrations of PL and PLP were measured by reverse-phase HPLC with fluorometric detection of PL- and PLP-semicarbazones (109).

**Statistical analyses.** Data are presented as means ± SEM of multiple experiments done at separate times and are expressed as pmol vitamin B-6 /mg protein. Differences between PN:PNG ratios in treatment medium from before and after incubation periods were detected using a one-way analysis of variance (115). Differences in intracellular concentrations of vitamin B-6 were examined using a one-way analysis of variance with Student-Newman-Keuls pairwise comparison test. Differences in PNG uptake and metabolism in the presence of lactose were analyzed by a two-way analysis of variance using concentrations of PNG and lactose as factors. Pairwise comparisons were performed using the Student-Newman Keuls test. The aforementioned statistical analyses were performed using SigmaStat statistical software (Jandel Corporation, San Rafael, CA).

## Results

**Uptake of PNG as a function of concentration.** PNG uptake in Caco-2 cells was examined at concentrations ranging from 0.5 μmol/L to 500 μmol/L (Figure 4-1). After 30 min at 37 °C, PNG was first detected in the intracellular compartment of the cells at 5 μmol/L, whereas no PNG was detected below this concentration. The intracellular concentration of PNG significantly increased ($P<0.0001$) with increasing concentrations of PNG in the incubation media over 30 min. PNG concentrations between 0-50 μmo/L were taken up in a saturable process (Figure 4-1A), while uptake of

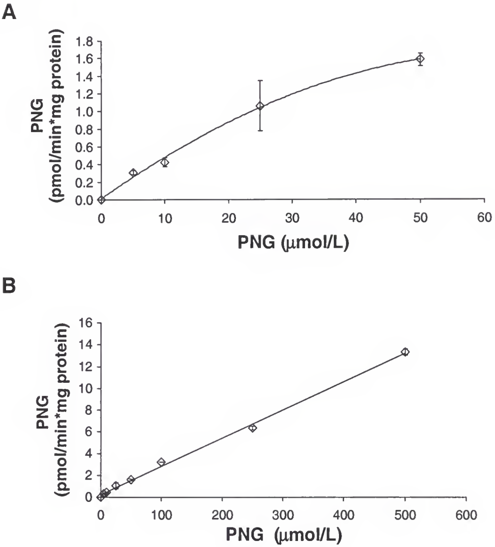


Figure 4-1. Pyridoxine-5'-β-D-glucoside (PNG) absorption by Caco-2 cells as a function of concentration after 30 min incubation. Absorption of PNG is saturable at ≤ 50 μmol/L (A) and non-saturable at >50 μmol/L (B). Each data point represents a mean of at least two replicates from 3 independent experiments. Data are presented as means ± SEM.

PNG at concentrations greater than 50 μmol/L was passive (Figure 4-1B).

**Uptake of PNG as a function of time.** PNG uptake was tested at 1, 2.5, 3, 5, 10, 15, 30, 60, and 120 min with 200 μmol/L PNG in the treatment media, which was a concentration that yielded a high intracellular concentration of PNG after a 30 min incubation (Figure 4-2). PNG uptake increased linearly up to 15 minutes and after that appeared to plateau.

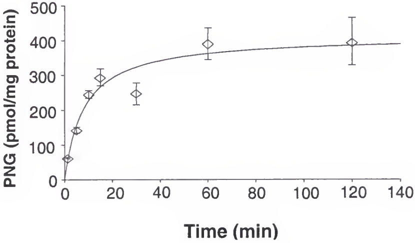


Figure 4-2. Absorption of pyridoxine-5'-β-D-glucoside (PNG) at 200 μmol/L by Caco-2 cells as a function of time. Each data point represents at least two replicates from three independent experiments. Data are expressed as means ± SEM.

**Distribution of intracellular B-6 vitamers in Caco-2 cells.** The determination of intracellular concentrations of vitamin B-6 in Caco-2 cells without added PNG revealed that most of the vitamin B-6 inside the cell was in the form of pyridoxine (Figure 4-3). Increasing concentrations of PNG in the treatment media did not significantly change the intracellular concentrations of PN, PLP, PM, or PMP.

**PNG hydrolysis by Caco-2 cells.** Activity assays for lactase were done on the total membrane fraction isolated from ultracentrifugation (200,000 x g, 30 min) of Caco-2 cell crude homogenates. Lactase activity for Caco-2 cells between 7-9 days post-confluency was 332 ± 11 nmol/h*mg protein. PNG hydrolytic activity was not detected in the cytosolic fraction of Caco-2 cells. PNG hydrolysis by lactase was measured by the comparison of PN:PNG ratios in the treatment media on top of the cells before and after the incubation period (Table 4-1). PN:PNG ratios did not significantly change from preto post-incubation in experiments using 25, 50, or 100 µmol/L PNG in the treatment media.

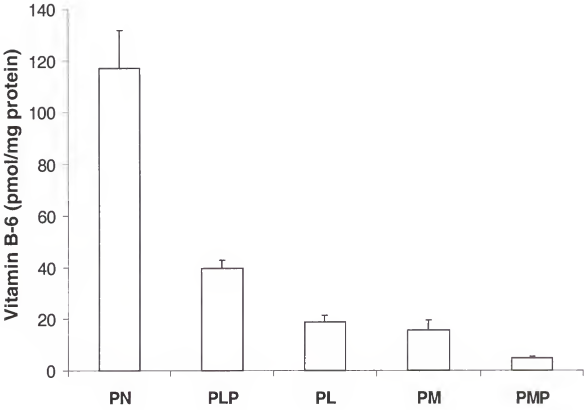


Figure 4-3. Distribution of intracellular concentrations of vitamin B-6 in Caco-2 cells without added PNG. Abbreviations: pyridoxine (PN), pyridoxal-5'-phosphate (PLP), pyridoxal (PL), pyridoxamine (PM), and pyridoxamine-5'-phosphate (PMP). Bars represent means ± SEM from at least 3 replicates from 4 independent experiments.

**Effect of lactose on PNG uptake and metabolism.** The effect of lactose on PNG uptake and metabolism was examined by incubating cell monolayers with 0, 25, 50, 100, and 200 µmol/L PNG with 0, 75, or 150 mmol/L lactose for 30 min (Figure 4-4). Intracellular PNG concentration significantly increased as the concentrations of PNG added to the cells increased. The presence of lactose, at any concentration, did not significantly change the intracellular concentration of PNG. However, intracellular concentrations of PL were significantly lower (P=0.01) in cells incubated with 75 and 150

Table 4-1. Pyridoxine to pyridoxine-5'-β-D-glucoside (PN:PNG) ratios in treatment media before and incubation period.

| PNG (μmol/L) | PN:PNG pre-incubation[1] | PN:PNG post-incubation | PN:PNG Mean change |
|---|---|---|---|
| 25 | 0.0020 ± 0.0003 | 0.0025 ± 0.0005 | 0.0006 ± 0.0006 |
| 50 | 0.0018 ± 0.0001 | 0.0020 ± 0.0002 | 0.0462 ± 0.0460 |
| 100 | 0.0017 ± 0.0001 | 0.0017 ± 0.0003 | 0.0004 ± 0.0002 |

[1] Incubations were done for 30 min at 37 °C. Values within column were not signifcantly different and the post-incubation ratio was not significantly different from the pre-incubation ratio.

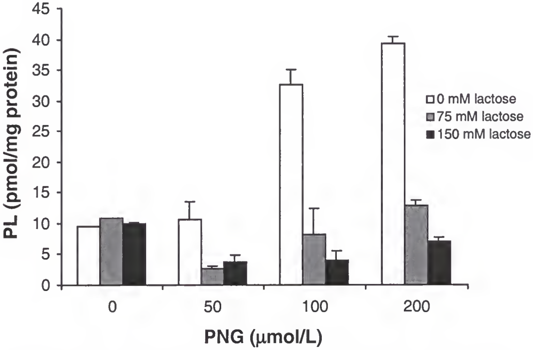


Figure 4-4. Effect of lactose on the intracellular concentrations of pyridoxal (PL). Increasing concentrations of lactose significantly decreased the intracellular concentration of PL. Bars represent means ± SEM of at least 2 replicates from 2 independent experiments.

mmol/L lactose than cells treated with no lactose. There were no significant differences in intracellular PLP concentrations in cells treated with lactose.

**Effect of sodium on PNG uptake.** On the basis of the hydrophilic structure of PNG, one would predict that it would be difficult for the molecule to pass through the

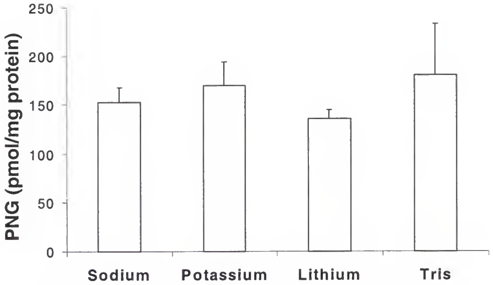


Figure 4-5. Effect of sodium and other monovalent cations on the absorption of pyridoxine-5'-β–glucoside (PNG) in Caco-2 cells after a 30 min incubation. Bars represent means ± SEM of three replicates from 2 independent experiments.

hydrophobic lipid bilayer of the intestinal epithelial cell plasma membrane. This suggests that the absorption of PNG could be facilitated by a carrier. To determine whether PNG uptake was mediated through a sodium-dependent transporter, the sodium in the Krebs-Ringer buffer was replaced by potassium, lithium, and Tris (hydroxymethyl) aminomethane-HCl to create a solution of equivalent ionic strength (Figure 4-5). PNG was added to each of the treatment media at 100 µmol/L and uptake was measured over 30 min. Intracellular concentrations of PNG were not significantly different (P=0.39) among the cells incubated in the sodium, potassium, lithium, or Tris treatment media.

## Discussion

The results from this study provide new insight into the intestinal absorption and metabolism of PNG. Vitamin B-6 uptake and metabolism were not examined in any intestinal epithelial cell culture prior to this investigation. Caco-2 cells function similarly to enterocytes in the small intestine, which permit the close examination of PNG uptake and metabolism.

PNG was taken up by the Caco-2 cell monolayers in a pattern that is consistent with passive diffusion. However, at low concentrations of PNG (5-50 μmol/L), the uptake of PNG displayed a saturable component. Intracellular concentrations of PNG increased linearly as the concentration of PNG (>50 μmol/L) provided to the cells increased. The range of PNG added to the cells encompasses an amount of PNG that would be consumed daily in the diet. Average dietary intake of vitamin B-6 by the U.S. population is 1.7 mg/d (NHANES III). Although food selection ultimately determines the percentage of vitamin B-6 derived from PNG, PNG contributes approximately 15% of total vitamin B-6 intake, which corresponds to ~200 μg PNG daily. This amount (~200 μg) divided equally over three meals yields the consumption of ~65 μg for each meal. Caco-2 cells incubated with 100 μmol/L (~66 μg) PNG took up this amount by passive diffusion. This suggests that absorption of dietary PNG in humans is mostly by passive diffusion. Absorption of smaller quantities of PNG may be facilitated by a carrier. This pattern of absorption is similar to that observed in the intestinal absorption of folate and many other nutrients. At low physiological concentrations of folate, the vitamin is absorbed by a pH-dependent, $Na^+$-independent carrier mediated transport system, while pharmacological concentrations of folate are absorbed by a diffusion process (138,139). The exchange of sodium for other monovalent cations did not change PNG uptake by Caco-2 cells; however, the concentration of PNG used in these experiments was in the range where passive diffusion, and not carrier-mediated absorption would predominate.

Collectively, the results of these studies suggest that physiological concentrations of PNG are taken up by a $Na^+$-independent process that is similar to the absorption of PN as previously determined rat small intestine (52,140). This observation is also consistent with a recent report of PN uptake by cultured opposum kidney (OK) cells (141). However, we cannot reconcile the difficulty that PNG, as a very hydrophilic molecule, would encounter in crossing the highly charged and hydrophobic plasma membrane. There is also recent evidence that other glycosylated compounds derived from the diet such as quercetin β-glucosides either interact with or are transported by a sodium-dependent glucose transporter (SGLT) (142-144). Although the absence of sodium did not cause a significant decrease in PNG uptake, we cannot rule out the possibility that PNG is taken up by a carrier mediated process. The transport mechanism is further confounded by the dual role ascribed to LPH. In a recent letter to the editor, Arts and colleagues briefly discussed the observation that LPH hydrolyzes β-glucosidic bonds and is thought to transport the aglycones in a $Na^+$-independent process (145). While this may explain the transport of PN from PNG into the cell, the process by which the intact glucoside, PNG, enters into the cell remains unclear. It is interesting to note that the detection of the intact glucoside inside the Caco-2 cells further supports previous observations made by this laboratory that PNG can be absorbed intact (7,10).

As indicated in Figure 4-3, the most abundant form of vitamin B-6 inside Caco-2 cells is pyridoxine. This was not wholly unexpected since these cells were maintained in DMEM that contained 4 mg/L of pyridoxine-HCl as the source of vitamin B-6. Consequently, it was difficult to measure the small changes that likely were occurring in intracellular PN concentrations in response to increasing concentrations of PNG. However, even the smallest change in intracellular PNG concentrations was easily detected because PNG is not present in DMEM, and there is no *in vitro* synthesis of PNG. The ratio of PN:PNG and its change over time provides a rough estimate of the PNG that is hydrolyzed at the apical surface of the cells. We did not observe any significant changes in these ratios and there are several complicating factors that might affect this measurement. Once hydrolyzed, PN could be taken up by the cell and metabolized or released into the treatment medium. Alternatively, over the incubation period, intracellular PN might efflux out of the cell into the treatment medium. The PN measured in the treatment medium could contain PN originating from the hydrolysis of PNG and that released from intracellular pools. We did however, obtain indirect evidence of PNG hydrolysis. Intracellular concentrations of PL significantly increased as PNG concentration increased in the treatment medium (Figure 4-4). PN taken up from the hydrolysis of PNG would be metabolized inside the cell to form PL and PLP. PL is reported to the major metabolic product of the small intestine released into portal blood (134). Since intracellular PL was present in relatively low concentrations in the Caco-2 cells without added PNG, small increases in PL were easily detected. We did not measure the concentration of PL in the treatment media after the 30 min incubation, but we suspect that intracellular PL would efflux into the media.

The addition of lactose to the treatment media did not appear to have a direct effect on the uptake of PNG. However, we have evidence that intracellular vitamin B-6 metabolism was changed, perhaps as a consequence of a reduction in PNG hydrolysis at the cellular surface. In the presence of lactose, LPH is inhibited with regard to PNG hydrolytic activity. Intracellular concentrations of PL significantly declined with increasing lactose concentrations. The concentration of 150 mmol/L was chosen since it is the approximate concentration of lactose in milk. At 150 mmol/L and half this concentration, 75 mmol/L, intracellular PL was significantly reduced in cells incubated with PNG. One possible explanation for this observation is that since PL is the greatest intestinal vitamin B-6 metabolite released into portal circulation (134), its concentration inside the cell might be most dramatically affected if the metabolic flux of PN to PL is reduced. Although PL concentrations declined, PLP concentrations did not significantly change in the present study. Our results are consistent with those of Middleton (146).

Under well-nourished or saturated conditions (high PN concentrations) PLP concentrations did not increase in the rat intestine, likely due to potent product inhibition by PLP on pyridoxine:pyridoxamine 5'-phosphate oxidase that is observed in the liver (147).

In summary, this study provides *in vitro* evidence that PNG is taken up intact by intestinal epithelial cells in a Na-independent process that is saturable at low concentrations and passively absorbed at concentrations >50 µmol/L, which is similar to the absorption of PN. Lactose diminished the metabolic utilization of PNG in Caco-2 cells, which might translate into a reduction of the *in vivo* bioavailability of PNG.

# CHAPTER 5
# SUMMARY AND CONCLUSIONS

The preceding research describes novel observations that extend our understanding of pyridoxine-5'-β-D-glucoside bioavailability, with particular focus on its absorption, hydrolysis, and metabolism in the small intestine. The following discussion integrates the data presented in the three previous chapters to provide a comprehensive view of the absorptive process of PNG and how it relates to its bioavailability.

Based on previous results from this laboratory, a decrease in dietary vitamin B-6 concentration significantly increased the intestinal hydrolysis of PNG by cytosolic PNG hydrolase (12,13). This inverse relationship between an induced vitamin B-6 deficiency and intestinal cytosolic PNG hydrolase activity was observed in both rats (13) and guinea pigs (12). Chapter 2 reports the results of two studies that were designed to examine this observation more closely. Although a vitamin B-6 deficiency was achieved, as assessed by a significant reduction in plasma and liver PLP concentrations, the effect of vitamin B-6 deficiency on cytosolic PNG hydrolase activity was not reproducible. There was a trend toward an increase in *in vitro* PNG hydrolytic activity as catalyzed by cytosolic PNG hydrolase and brush border membrane LPH as dietary vitamin B-6 increased. In this respect, it appears that there is not a strong compensatory upregulation of the hydrolytic activity that could enhance the metabolic utilization of PNG in a time of dietary vitamin B-6 deficiency.

In an attempt to further study this indirect relationship between dietary vitamin B-6 and PNG hydrolytic activity similar conditions, controlled feeding studies using pyridoxine-defined diets were done in rats to explore potential differences between studies done by this laboratory in the past and present. The investigation done by Nakano et al. (13) used older rats weighing ~200g and fed PN-defined diets for 2 wk. Preliminary studies that were used as a foundation for the present work initially examined the effects of age and duration of diet feeding on intestinal PNG hydrolytic activity and found no significant effects of rat age or length of study period.

One other notable difference between the present studies and previous studies done by this laboratory is in the diet that was fed to the rats. We speculated there to be an effect of basal diet formulation (AIN-93G or AIN-76A) on PNG hydrolytic activity. The present studies consistently fed the AIN-93G diet with different concentrations of pyridoxine to rats over a 5 wk period to change their vitamin B-6 nutritional status. The AIN-93G diet was formulated to reduce symptoms such as tooth decay and kidney calcification that were associated with chronically feeding the AIN-76A diet to rats (107). As discussed in Chapter 2, the carbohydrate source in the AIN-76A diet is sucrose, which was replaced with starch in the AIN-93G diet. The starch based AIN-93G diet theoretically could support the energy requirements of the gut microflora. Starches and oligosaccharides that are not completely hydrolyzed and absorbed in the small intestine travel to the large intestine where the resident colonic microflora ferment the undigested starch to salvage metabolic energy. A thriving population of gut microflora synthesizes short chain fatty acids, proteins, growth factors, and vitamins such as vitamins B-6 and K, which could be absorbed by the colon (108, 148). AIN-93G also is supplemented with trace minerals that are absent in the AIN-76A diet (107) and although these differences exist, the diets are intended to be nutritionally equivalent. In spite of this, we observed significantly greater rat growth and PNG hydrolytic activity in the cytosolic and total membrane fractions in rats fed the AIN-93G in comparison to rats fed AIN-76A, regardless of dietary PN concentration. There is a lack of information on the effects of feeding AIN-93G and AIN-76A diets side-by-side in controlled nutritional studies. At present, it is unclear what specific dietary component(s) influence these outcome measures.

The purification and partial kinetic characterization of LPH, presented in Chapter 3, revealed that LPH was the enzyme in the brush border that was able to catalyze the hydrolysis of PNG. The primary function of LPH is to catalyze the hydrolysis of dietary lactose and the hydrolytic activity toward PNG had not been previously reported. The $K_m$ value calculated for lactose was 16 times greater than that calculated for PNG. In considering the quantity of PNG that is introduced to the intestine daily (~15% of the total vitamin B-6 intake of 1.7 mg/d), the enzyme is well-suited to catalyze PNG hydrolysis. However, the enzyme is not likely to be working efficiently and would catalytically prefer the hydrolysis of lactose to PNG. Not surprisingly, lactose was found to be a competitive inhibitor of PNG hydrolysis. This leads us to the questions of whether PNG bioavailability is reduced by the composition of a mixed meal, i.e. dairy products containing lactose consumed concurrently with fruits or vegetables containing PNG, or if PNG bioavailability is diminished in individuals with lactose intolerance or lactase insufficiency.

In addition to the PNG hydrolytic function of LPH, we also observed yet another novel function of mammalian LPH. In the presence of both PNG and lactose, LPH exhibits glucosyl-transferase activity *in vitro*. We measured not only the hydrolytic product of PNG, PN, but also a secondary product, PN-disaccharide. The significance of this reaction *in vivo* is not completely understood, but may further contribute to a reduction in PNG bioavailability.

Perhaps the most noteworthy finding of this research is that LPH was able to hydrolyze PNG and it is apparently responsible for a large proportion of the intestinal hydrolysis of PNG. After some preliminary feeding studies (data not shown) it soon became evident that the activity measured in the brush border membrane fractions of intestinal mucosa was just as important as the hydrolysis measured in the cytosolic fraction. Chapter 2 also described the calculation of the relative contribution of LPH and cytosolic PNG hydrolase to total PNG hydrolysis measured in the small intestine. LPH is the only β-glucosidase in the brush border membrane and any hydrolysis measured in the brush border membrane is due to the activity of LPH. It was estimated in the rat that 50-60% of the PNG hydrolysis in the small intestinal mucosa was catalyzed by brush border LPH. In comparison, cytosolic PNG hydrolase was found to catalyze only 10% of the hydrolysis of PNG in the rat intestine. It was initially thought that the hydrolysis of PNG was exclusively catalyzed by cytosolic PNG hydrolase, but we now have strong evidence to support that PNG is hydrolyzed by extracellular (LPH) and intracellular (PNG hydrolase) enzymes and that LPH is responsible for most of this hydrolysis. Therefore, based on the results obtained from the present studies in rats and Caco-2 cells, PNG bioavailability is greatly dependent on a functionally active LPH.

Results from the study of PNG absorption and hydrolysis by Caco-2 cells presented in Chapter 4 support previous data generated by this laboratory. While PNG can be hydrolyzed in the small intestine to form PN and glucose, it is not a complete hydrolytic process and PNG can be absorbed as the intact glucoside (7,10,11). PNG provided to Caco-2 cells was rapidly taken up in a saturable process at concentrations less than 50 μmol/L, and taken up in a non-saturable, passive diffusion process at concentrations greater than 50 μmol/L. We also collected preliminary data that suggested that there is little paracellular absorption of PNG (data not shown). Pilot experiments were done with the Caco-2 cells grown on permeable membrane inserts and intercellular permeability was tested by measuring the passage of phenol red, a marker for paracellular permeability. Trace amounts of phenol red, a low molecular weight compound (MW 352), were detected on the basolateral side of the Caco-2 cell monolayer. PNG has a MW that is very similar to phenol red (MW=331). Therefore, we believe that PNG absorption proceeds mostly via transcellular route; however, absorption by the paracellular route cannot be excluded. Application of these data to human dietary intake of PNG indicates that PNG derived from the diet is mostly absorbed by passive diffusion; however, absorption of PNG in individuals consuming a diet limiting in foods of plant origin might occur through carrier-mediated transport.

In summary, the results from the studies discussed in the previous three chapters indicate that 1) PNG can be absorbed intact, as it was detected intracellularly in the Caco-2 cells, 2) PNG can be enzymatically hydrolyzed to release free PN at the brush border membrane by LPH, or by cytosolic PNG hydrolase, and 3) *in vitro* hydrolysis is markedly reduced in the presence of lactose. We have constructed a model of PNG absorption based on the results from our laboratory (Figure 5-1). This model incorporates many years of PNG bioavailability research and presents our current understanding of PNG intestinal hydrolysis and absorption.

Future research should address the possible reduction in PNG bioavailability in individuals with lactose intolerance. Lactose intolerance, or lactase insufficiency, affects 75% of the population worldwide (101) and this intestinal condition could compromise vitamin B-6 nutritional status in populations consuming a vegetarian based diet. Conversely, in individuals with lactase persistence, PNG bioavailbility may be increased. However, lactose co-ingested with foods of plant origin providing PNG might decrease the amount of vitamin B-6 derived from PNG. These questions remain to be answered and further investigation is warranted.

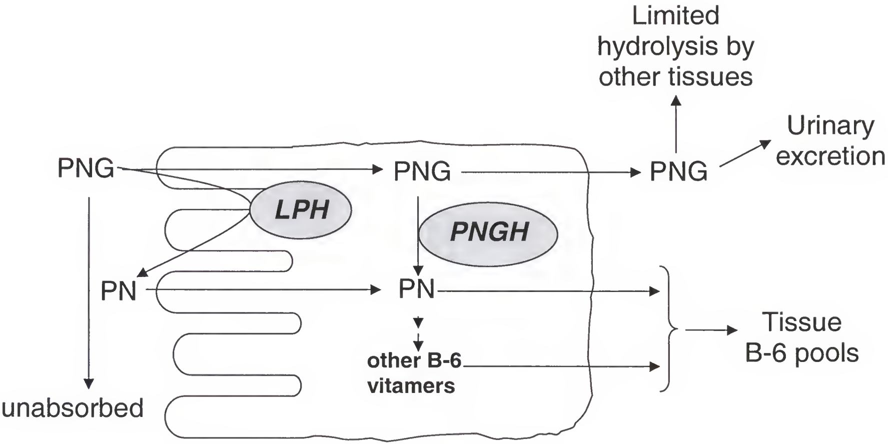


Figure 5-1. Illustration of PNG hydrolysis, absorption, and metabolism in the small intestine. Abbreviations used: PNG, pyridoxine-5'-β-D-glucoside, PN, pyridoxine, LPH, lactase-phlorizin hydrolase, PNGH, PNG hydrolase.

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